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An Investigation Into The Biological Responses Induced Following Oral Exposure To Silver nanoparticles

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An investigation into the biological responses induced following oral exposure to silver nanoparticles

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A thesis submitted to Dublin Institute of Technology

For the degree of Doctor of Philosophy

School of Physics

Dublin Institute of Technology

Kevin Street, Dublin 8

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Abstract

This study aims to investigate the biological responses induced by silver nanoparticles (AgNP) following oral exposure. The commonality of AgNP in consumer goods highlights the need for a thorough investigation into the interaction with and subsequent responses evoked in living systems following exposure. Firstly, the potential interaction with and effect of biofluid components, namely cholic acid (CA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA), on AgNP toxicity was investigated. Two cell lines corresponding to organs related to the biofluid components, HepG-2 a hepatocellular carcinoma derived from liver tissue and Hep2 an epithelial cell line, were employed. Physicochemical and cytotoxic screening was performed and the ability of biofluid components to modify AgNP cytotoxicity was explored. No alteration to the physicochemical characteristics of AgNP by biofluid components was observed; however their addition resulted in altered AgNP toxicity. Greater reactive oxygen species (ROS) induction was noted in the presence of CA and DCA. UDCA demonstrated no modification of toxicity in HepG-2 cells however significant modification was observed in Hep2 cells. It was concluded that biofluid components can modify AgNP toxicity but is dependent on the biofluid component itself and the location where it acts.

As inhalation is the most common route of nanoparticle entry and given the close proximity to the GI tract, the tendency of cross exposure between the two is prevalent. As such the next line of investigation involved the potential toxicity of AgNP to A549 alveolar epithelial carcinoma cells and the influence of a major component of lung surfactant dipalmitoylphosphatidylcholine (DPPC) on toxicity. This follow up investigation revealed that exposure generated low levels of oxidative stress and a reduction in cell viability. While the presence of DPPC caused no influence on viability studies its presence increased ROS formation and significantly modified the inflammatory response generated by AgNP exposure. These findings suggest a possible interaction between AgNP and DPPC causing particles to become more reactive thus increasing oxidative insult and inflammatory response within A549 cells.

The final investigation in this report was on the biological effects of AgNP on the innate immune response of circulating white blood cells. This study determined the ability of

AgNP to induce an inflammatory response in THP-1 monocytes by measuring AgNP stimulated gene expression of the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α). A further study on monocytes extracted from a cohort of blood samples, was carried out to compare the inflammatory response to THP-1 monocytes. Finally ELISAs were performed on supernatants of THP-1 monocyte cultures to test for the activation of pro-IL-1 β a key mediator of the inflammasome complex. The findings clearly demonstrate AgNP can significantly up-regulate pro-inflammatory cytokine gene expression in THP-1 and primary human blood monocytes, with IL-1 β release by inflammasome involvement indicating AgNP can result in an immunologically active state.

Declaration

I certify that this thesis, which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, to lend or to copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged

Signature _____ **Date** _____

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Chapter 1

Introduction and Overview

1.1 Introduction to Nanotechnology

The origins of modern nanotechnology begin with the physicist Richard P. Feynman and his lecture in 1959 entitled “There’s Plenty of Room at the Bottom”. In the lecture Feynman described that it was not the laws of physics which prohibited the manipulation of atoms or molecules but the methods and tools available at that time (Feynman 1959). Despite Feynman’s conjecturing, it was not until 1974 that Norio Taniguchi first used the term nanotechnology stating that “Nanotechnology mainly consists of the processing, separation, consolidation and deformation of materials by one atom or one molecule” (Taniguchi 1974). From that time the definition of nanotechnology has been expanded and changed. In October 2011, the European Commission published a report which defined a nanomaterial as;

“A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate, and where for 50% or more of the particles in the number size distribution, one or more external dimensions is in the range 1-100nm

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold may be replaced by a threshold between 1-50%

By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1nm should be considered as nanomaterials.”

This recommendation of a definition for a nanomaterial was to be used for materials that require special provisions such as risk assessment (European Commission, 2011).

While this definition aimed to encompass a general nanomaterial definition, there are numerous definitions detailing the parameters of what classifies a nanomaterial within certain sectors such as cosmetics, food and biocides. This in itself presents a lot of

confusion regarding the definition of a nanomaterial as the context in which the nanomaterial is presented must be taken into account (European Commission, 2009; 2011; 2012).

This EU Commission definition has generated a lot of controversy and was considered too broad by some industries, too narrow by others and used inappropriate thresholds. As a result this definition is currently under review as to whether the size number distribution threshold of 50% should be increased or decreased. This review is due to conclude in 2016 following consultation with stakeholders regarding its findings towards the end of 2015 (European Commission, 2015).

1.2 Potential of Nanotechnology

Nanotechnology has often been described as a new frontier of this century with many different applications in various fields including textiles, electronics, medicine, cosmetics and food, demonstrated graphically in figure 1.1 (Sozer & Kokini, 2008; Bouwmeester *et al*, 2009). Between March 2006 and February 2008 the number of consumer products containing nanoparticles had tripled with more than a thousand commercial products identified (Powell *et al*, 2010). It was discovered during this time that over 200 manufactures marketed their products as “nano-products” and of these 9% were food and beverage while health and fitness accounted for 60% (Sozer & Kokini, 2008). A recent report stated that the global nanotechnology market was worth an estimated \$26 billion in 2014 and is predicted to reach approximately \$64.2 billion by 2019 (McWilliams, 2014). The inventory of consumer products currently on the market in 2015 using nanotechnology has increased substantially to 1,628 a 24% increase from 2010. This inventory allows the public to browse the products currently available and also provides details on the companies behind these nano-products. Of the 1600+ products listed, 117 accounts for food and beverage based products with health and fitness making up the largest number of products at 907. Silver proves to be the most popular nanomaterial of choice with 483 of the 1628 products are listed as containing nano-silver (Nanotechproject, 2013). This exponential growth in nano-based products and increased consumer exposure will only continue to grow and highlights the need not only for strict regulation but also for a complete risk assessment on the impact of exposure. This work will examine the biological effects of exposure to nanoparticles on

humans, ultimately contributing to an on-going project into the development of a complete and detailed risk assessment of the potential harm resulting from exposure to nanoparticles. While there are a number of different routes of entry, this project will focus on the oral route of exposure and thus will focus on the applications of nanotechnology where oral exposure to nanoparticles is a requirement or a concern.

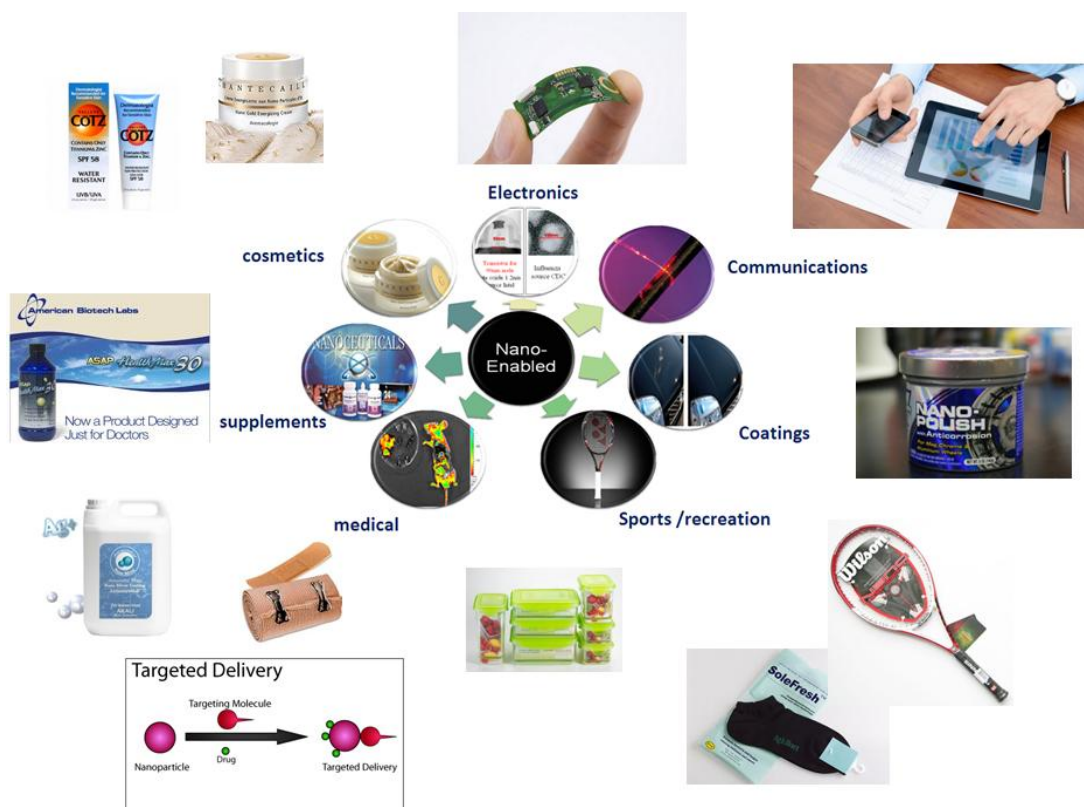


Figure 1.1 Applications of Nanotechnology and some examples of the nano-enabled products available

1.3 Application of nanotechnology in areas of industry where oral exposure is essential or a concern

The applications for nanotechnologies are numerous and the likelihood of exposure to humans is becoming increasingly significant. Their inclusion into a variety of everyday products only increases the incidence of contact and raises questions regarding potential hazards of exposure. While their inclusion can provide various benefits there is the potential to produce undesirable biological effects. Oral exposure provides one of the

easiest routes of nanoparticle entry be it through food additives, drug delivery (ingestion) or products such as deodorants, fabrics and bedding (inhalation), and highlights this route as one of the most common entry points and one in which any risk assessment regarding nanoparticle safety must be focused (Sekhon, 2010; Boisseau & Loubaton, 2011; Cerkez *et al*, 2012).

1.3.1 Application of Nanotechnology in the Food Industry

Nanotechnology is nothing new to the food industry with certain well established products employing a form of nanotechnology to reduce fat content of for example mayonnaise by emulsifying fat droplets in nano-sized water droplets without effecting taste. More recent uses of nanoparticles include food supplements, additives, novel foods, flavourings and packaging (Smolkova *et al*, 2015). The presence of nanoparticles in food can be unintentional as well as intentional with nanoparticles used in all aspects of the food industry from use as pesticides or fertilizers to veterinary drugs. The presence of nanoparticles in food packaging as a means of detecting food spoilage or monitoring temperature and moisture, present another route of unintentional nanoparticle presence in food through leaching. Nanoparticles such as titanium dioxide (TiO₂) are added to powdered food as anticaking agents in products such as powdered milk and sugar as well as its use as a white colouring for confectionary and white coloured sauces and dressings (Lomar *et al*, 2002; Smolkova *et al*, 2015). Silver nanoparticles (AgNP) are used as antibacterial agents in food products and can be used as a detection method for food spoilage known as “e-noses”. Another aspect is the use of AgNP in gels to coat food such as vegetables preventing water loss and their incorporation into cellulose pads to prevent microbial growth when placed in contact with meat products including beef (Tung *et al*, 2014; Smolkova *et al*, 2015).

One of the most important aspects of nanotechnology in food is the ability to allow delivery of certain labile bioactive compounds such as antioxidants and vitamins. The encapsulation of such compounds in solid lipid nanoparticles can potentially increase their bioavailability and stability, and protect from degradation by various enzymes and acids in the GI tract. This application has the potential to improve overall health and can be applied to pharmaceuticals as well as natural bioactive compounds (McClements, 2013; Weiss *et al*, 2008; Yao *et al*, 2015).

A recent extensive research of nanotechnology in the food sector by the European Food Safety Authority (EFSA) revealed 633 nanotechnology applications with product names, related suppliers and described the physio-chemical characteristics of nanomaterials in the food, agriculture and feed sectors. 55 types of nanomaterials were identified and the most common applications were found to be in food additives or food contact materials (Peters *et al*, 2014).

1.3.2 Application of Nanotechnology in Drug Delivery

Nanotechnology is well established in drug delivery as an intelligent method of delivery maximising the targeted control of compounds while minimizing side effects. The application of nanotechnology in drug delivery aims to improve bioavailability of drug compounds, prolong their residence time in the body and allow for targeted delivery to affected sites while sparing healthy cells. Engineering polymeric nanoparticles is a growing area of drug delivery research and shows promise as a method of targeted drug release. These polymers can be produced to be biocompatible and are often biodegradable, allowing intelligent controlled release of a drug product while producing non-toxic by-products following degradation that are then easily eliminated from the body (York *et al*, 2008). Dendrimers are also a popular choice for drug delivery with drug molecules either encapsulated within the dendrimer or covalently attached to the surface. A number of characteristics including easy manipulation of size and attached functional groups, high penetration of cell membranes and a lack of immunogenicity make them a popular delivery vehicle (Yang *et al*, 2009; Siewiera & Labieniec-Watala, 2012; Safari & Zarneger, 2015).

Oral delivery of chemotherapeutic drugs is a growing area of interest in order to maintain a constant high level of exposure of chemotherapeutics to cancer cells while also improving patient comfort. This could provide a breakthrough in the available cancer treatments given intravenously which result in peaks above the maximum tolerable concentration and fast excretion from the circulation, thus limiting the therapeutic effects and resulting in serious side effects typically associated with current cancer treatment (O'Neill & Twelves, 2002; Feng *et al*, 2011; Jia *et al*, 2013; Mei *et al*, 2013). Dendrimers have been highlighted for potential oral delivery due to their ability to permeate intestinal epithelium enhancing drug absorption (Wiwattanapatapee *et al*,

2000). Polymeric micelles are another means of delivery providing increased solubility of drugs and improving oral bioavailability (Gaucher *et al*, 2010). Liposomes have also been highlighted as a possible carrier due to their potential protection of encapsulated drugs from intestinal degradation while increasing absorption (Moutardier *et al*, 2003; Mei *et al*, 2015). This method of cancer treatment provides many challenges in its design with numerous factors to be considered both physiologically and the particular properties required of the carrier. The treatment of cancer through oral drug delivery may not be too far off and highlights how the application of nanotechnology can be used to improve the treatment of diseases such as cancer and improve the quality of life of those affected.

1.4 Concerns of Nanotechnology

Everyday consumer products containing nanomaterials are becoming more widely available and easily accessible. As a result it is important to consider various questions raised regarding the health and environmental risks associated with these products. Together with potential risks, the concerns of the public must be addressed regarding these products. In the past not acknowledging the public view has led to problems with the introduction of new technologies, in particular regarding the introduction of genetically modified (GM) products (Gaskell *et al*, 1999). With the continued growth of nano-based products, it is important to take into account the public concerns at the beginning of product development in order to avoid a backlash similar to that observed with the introduction of GM products. Studies have been carried out in order to gauge public perception of nanotechnology and have concluded that general knowledge on this topic is very limited with many not understanding the term nanotechnology and what it implies. A study into the general public's perception on new food technologies including nanotechnology highlighted that consumer acceptance of a new technology cannot be assumed, and that consumers reactions to new technologies and the likelihood of acceptance varies with each individual and each technology. The main observations from this study indicated that stakeholders must carefully consider the methods involved in introducing new technologies well in advance of market launch and that transparency is paramount in building consumer confidence and acceptance of new technologies (Henchion, 2012). This study has highlighted that there is still clearly a

need for a detailed and complete risk assessment into the applications of nanotechnology, particularly nano-silver to build consumer confidence in the area.

The need for proper and strict regulation guidelines and testing are only now coming to the forefront with the exponential growth in the industry over the past couple of years. In April 2015 the US Environmental Protection Agency (EPA) was forced to stop the sale of two nano-silver based products for use in hospitals as sterile agents due to unsubstantiated claims made by the company that these products “can swiftly eradicate all micro-organisms and keep surfaces free of colonization for up to a full year” (Nano Defence Solutions Inc). Under new federal law filed by the Center for Food Safety any product that contains a pesticide as an active ingredient must be registered with the EPA and determined to be risk free when used as per the labels instructions. The EPA now acknowledge nano-silver antimicrobial products should be regulated as new pesticides and that products must be regulated and safety tested by independent experts in order to safeguard the public from unregulated nano-products on the market (Center for Food Safety, 2015).

1.4 Nanosilver

Bulk silver is a white and brilliant metallic element that has been utilized for thousands of years due to its antimicrobial activity, high thermal and electrical conductivity and its low contact resistance (Nordberg *et al*, 1988). Usage of silver in its bulk form has a long history dating back thousands of years. Its use for the preservation of food and purification of water in ancient times has been well documented with large silver vessels used for the storage of wine and water. During World War I silver was used to treat wounded soldiers and prevent infection prior to the discovery of antibiotics (Russell & Hugo, 1994; Chen & Schluesener, 2008). Although it has well described healing and preservative properties, prolonged exposure to silver has been associated with irreversible pigmentation of the skin known as argyria (Rosenman *et al*, 1972; Marshall & Schneider, 1977; Gulbranson *et al*, 2000; White *et al*, 2003). Apart from this condition silver was believed to be relatively non-toxic and in the advent of nanotechnology, manufacture of silver on a nano-scale has become a rapidly expanding area of development.

Nanosilver is probably one of the most highly used nanomaterials on the market today with applications ranging from antibacterial textiles and medical devices to food packaging, cosmetics, water treatment and electronics. Compared to bulk silver, nanosilver displays different physicochemical properties and can exhibit different biological activities (Zhai *et al*, 2006; Dawy *et al*, 2012; Greulich *et al*, 2009). Probably the most important property of nanosilver and the one that makes it so appealing is its antimicrobial activity. Its ability to inhibit microbial growth has made its use in the medical sector invaluable with dressings, bandages and catheters to name a few embedded or coated with nanosilver to prevent infection and sepsis (Cheng *et al*, 2004). This antimicrobial property has now been applied to everyday consumer products from laundry detergents and water purificants to paints and even clothing. It has been reported that the antimicrobial effect of AgNP result from their ability to interfere with membrane permeability and redox-cycling in the cytosol of bacteria by attaching to the cell membrane, causing accumulation of intracellular radicals and if small enough, eventually entering the cell causing DNA damage (Liang *et al*, 2010). Staying within the medical sector nanosilver also has uses diagnostically. In the area of bio-imaging and bio-sensing the plasmonic properties of nanosilver are used to improve diagnostic mechanisms. The attachment of nanosilver to biosensors reduces the interference of the buffer solution it is suspended in and produces greater resolution. For bio-imaging compared to the fluorescent dyes commonly used, nanosilver can be detected by numerous microscopy techniques and can be used to monitor dynamic biological events for extended periods of time due to its photostable property (Sotiriou & Pratsinis, 2011).

1.5 This Thesis

This study focuses on AgNP, commonly used across a variety of industries due to their antimicrobial properties. The reason being that while there are benefits which have led to growth and innovation within the area of nanotechnology, there is still a lack of regulation and a gap in the knowledge regarding the fate of AgNP following exposure, the interaction with biological entities and how this can ultimately affect the responses produced. It is important to establish the interaction with and potential toxic effect on biological systems in order to produce a complete and detailed risk assessment, not to hinder the development of the industry but to provide knowledge so that further innovation utilizing AgNP can be carried out in an informed, regulated and safe manner.

This study is part of an on-going Science Foundation of Ireland (SFI) funded project with the overall aim to develop a risk assessment for oral exposure to silver nanoparticles from food products. From the work performed over the last four years it is hoped that the knowledge gained can actively contribute to this risk assessment and provide insight into the effects of nanoparticle exposure on living systems, furthering the understanding of the toxic profile of AgNP.

Specifically this thesis aims to establish nanoparticle interaction with biological entities, namely biofluids, associated with the GI tract and the lung, two organ systems affected by oral exposure and how this interaction may affect the response produced. This study does not end here, as nanoparticles do not remain in initial exposure sites, but have the ability to translocate through the body moving to different biological compartments eventually reaching circulatory system. The final part of this investigation is to determine the immunological significance of AgNP on circulating white blood cells and whether their presence can induce an innate immune response. An overview of the thesis is summarized in figure 1.2, and highlights the thought process of the thesis design, following the route of AgNP from initial oral exposure to eventual absorption into the bloodstream.

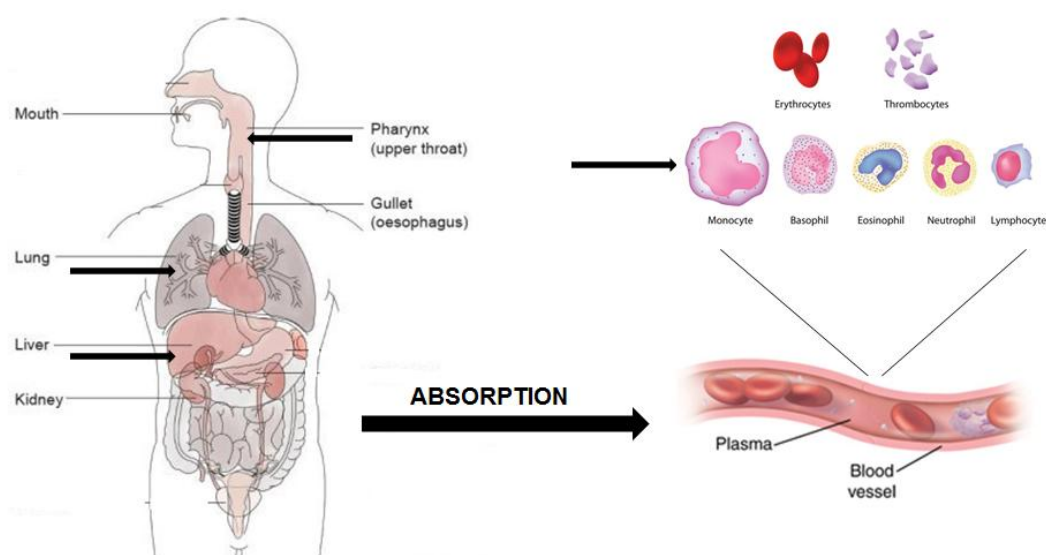


Figure 1.2 Summary diagram of thesis outline highlighting the main focus points following the potential route taken by AgNP following oral exposure. Arrows indicate areas of interest throughout the study

Before any toxicity studies can be performed it is first essential to carry out full physicochemical characterisation of the tested AgNP. This will provide important information and give insight into how AgNP will interact with different cells of the body following internalisation. This characterisation will be presented in chapter 4. Investigations into how AgNP interact with various biofluid components present in the body, such as bile acids and lung surfactant will be assessed. Their ability to modify the toxic profile of AgNP in the liver, larynx and lung all areas associated with oral exposure will be presented in chapters 5 and 6. In doing so this study aims to mimic real life exposure scenarios and determine the influence of certain biological entities, in this case components of biofluids, on the biological response induced following AgNP exposure. Following this an investigation into the effects induced by AgNP exposure following absorption into the circulatory system will be investigated. The potential effect of AgNP on the innate immune response of circulating white blood cells will be explored. First a monocyte cell line was used to determine pro-inflammatory cytokine gene expression induced by AgNP exposure, indicative of an inflammatory response. A further study on human monocytes extracted from a cohort of human blood samples was

carried out to compare the AgNP response with that observed in the monocyte cell line, all detailed in chapter 7.

The key research questions that are considered over the PhD are as follows:

- What are the physicochemical characteristics of AgNP and does the addition of biofluid components alter them?
- Are AgNP toxic to cells associated with oral exposure and does the addition of biofluid components modify the observed toxicity?
- Can AgNP exposure result in up-regulation of pro-inflammatory cytokine gene expression in THP-1 monocytes?
- Can AgNP exposure result in up-regulation of pro-inflammatory cytokine gene expression in primary human monocytes and how does this response compare to the response in THP-1 monocytes?
- Does AgNP exposure result in cleavage and activation of pro-IL-1 β , indicating a role for the inflammasome complex in AgNP response in circulating blood cells?

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Chapter 2

“Nanotoxicology”

2.1 Introduction

The previous chapter highlighted the potential of nanotechnology and its benefits as a technology across a wide range of industries, including medical, cosmetics and food with a focus on AgNP. Although it continues to be marketed as an advantageous technology improving the quality of products in the areas of health, food, technology and agriculture a number of concerns are being raised as to the impact of this technology on the environment and human health. The rapid growth of this industry and apparent lack of regulation has led people to question the possible harm associated with this technology. This chapter will discuss some of the issues associated with nanomaterial and nanoparticle exposure, the various routes of exposure and the risk associated with exposure to AgNP. It should be noted however that the benefits of nanotechnology greatly outweigh the negatives and so any risk assessment must be balanced so as not to stifle innovation and development.

2.2 Nanoparticle interactions with Biological Systems

The relationship between nanoparticles and biological systems is complex, with a variety of factors including physicochemical characteristics of the particle and the position within the body as well as the variety of proteins and biological entities present influencing the types of interactions and responses produced.

These intermolecular forces apart from dictating the biological response evoked by nanoparticle exposure are fundamental to life itself. Intermolecular interactions are required for the basic functioning of cells. Proteins, a fundamental components of cells, are formed by the formation of peptide bonds which link a number of amino acids together resulting in the production of a protein. Protein folding is important in determining function and occurs through several types of weak molecular interactions; hydrogen bonds, ionic bonds and van der Waals forces. Although weak, a large number of these bonds form allowing protein folding to occur and form a stable conformation.

A fourth force is also involved. Clustering of the non-polar hydrophobic side chains of the polypeptide towards the inside of the molecule will allow these side chains to avoid the aqueous cell environment thus further affecting protein shape (Alberts *et al*, 2002). In addition to protein formation and folding, intermolecular forces are required to allow proteins to carry out functions including binding specifically to ligands in order to carry out specific functions, for example antibody binding to specific bacterium marking it for destruction and removal from the body.

In addition to proteins, attractive forces are essential to DNA structure with hydrogen bonding between base pairs producing the distinctive double helical shape of the DNA molecule (Guerra *et al*, 2000). Haemoglobin is a complex protein structure made up of multiple protein subunits that requires the formation of covalent bonds such as disulphide bridges and hydrogen bonds to maintain stability following folding (Cooper, 2000; Berg *et al*, 2002). From the basic formation of the cell to construction of proteins to the reversible binding of oxygen to heme for transportation around the body, molecular interactions are essential for living systems and form the basis of life itself.

While it is clear that intermolecular forces are vital to life, forming the basis of cellular structure and function, it is important to consider the interaction between nanoparticles, cells and other biomolecules. As intermolecular interactions are fundamental to living organisms and dictate various processes, the same focus should be put on nanoparticle-biomolecule interactions as these may also dictate biological outcome following exposure.

The interaction between nanoparticles and various biomolecules including proteins has become an important topic in understanding the behaviour of nanoparticles following exposure. Not only is the type of biomolecule or location within the body a factor, but also the physicochemical characteristics of the nanoparticle can dictate the type of response induced (Lynch *et al*, 2009; Ehrenberg *et al*, 2009). Following entry into living systems a dynamic layer of biomolecules adsorb onto the nanoparticle surface and this coating is what cells “see” and interact with. Nanoparticles may become coated with proteins, lipids, sugars and other molecules impacting not only the properties of the nanoparticle itself but also the adsorbed biomolecules (Cukalevski *et al*, 2011; Bailes *et al*, 2012; Lesniak *et al*, 2012). Biological interactions have been shown to vary as a nanoparticle moves from one biological environment to another, subsequently changing

the composition of the surface coating with evidence suggesting the same nanoparticles can elicit different responses based on the particular composition of the surface coating (Maiorano *et al*, 2010; Lundqvist *et al*, 2011). With the variety of biomolecules present and the number of additional factors involved in nanoparticle-biomolecule interactions, it is becoming apparent that a need for mapping the surface coatings of nanoparticles in the relevant biological and cellular compartments, in order to understand the various interactions and possibly predict the responses produced is required (Lynch *et al*, 2007; Byrne *et al*, 2013).

It is safe to say that with the literature available nanoparticles will not exist as non-interacting entities within biological systems but will quickly become associated with a layer of biomolecules following entry and this surface coating will vary depending on a number of factors relating to the nanoparticle itself, the biological location and types of biomolecules encountered. It is this interaction that provides vital information into predicting nanoparticle behaviour and biological outcome, both of which must be addressed in toxicological research.

2.3 Exposure Routes

When considering the risks associated with contact to nanoparticles and nanomaterials one of the aspects to be identified first is exposure routes to the body. There are three main routes of entry that must be considered;

1. Skin
2. Inhalation
3. Oral Exposure

2.3.1 Skin

Dermal exposure to nanoparticles is becoming increasingly common particularly in the area of drug delivery where nanoparticle formulations have been incorporated into topical creams and lotions. The skin represents the main protection barrier of the body and provides first line defence against infection and environmental hazards. The barrier function of the skin is provided by the stratum corneum which is composed of highly differentiated keratinocytes. These keratinocytes contain a natural moisturising factor

which keeps the skin hydrated and are held together by a lipid lamellae layer preventing water loss and providing barrier permeability (Cork *et al*, 2009). While it has been previously demonstrated that titanium dioxide (TiO₂) and zinc oxide (ZnO) nanoparticles do not penetrate the skin, it has been noted that metallic nanoparticles of 10nm and less could penetrate the skin and deposit in the deepest layers of the stratum corneum (Baroli *et al*, 2007; Pflucker *et al*, 2001; Shulz *et al*, 2002; Cross *et al*, 2007). In relation to the penetration of AgNP into the skin, it was demonstrated that particles of 30nm and less can penetrate the skin eventually reaching the deepest layers of the stratum corneum and that in damaged skin this permeation is increased (Larese *et al*, 2009). It was suggested that a certain amount of particles dissolve and diffuse through the skin as elemental silver, this hypothesis is similar to a mechanism observed for nickel penetration where stratum corneum diffusible compounds are formed by the oxidation and solubilisation of particles on the skin surface and enter by an intracellular route (Hostynek *et al*, 2001).

2.3.2 Inhalation

Nanoparticle exposure via inhalation is probably the most common entry point for nanoparticles and because of this, targeted and controlled drug delivery via the lungs has become a popular area for development. Inhalation provides an easy entry route for engineered nanoparticles in nano-carrier systems for drug delivery, allowing an easy route for targeted, controlled and non-invasive delivery. Liposomes in particular have been the nano-carrier of choice in the development of improved and intelligent drug delivery systems (Varez-Lorenzo *et al*, 2009; Shum *et al*, 2001). A number of investigations into the mechanisms of nanoparticle uptake in the lung have provided information into the methods required for the formulation of potential carriers to induce uptake and the various access points. Entry may occur via a transcellular mechanism mediated by specific receptors, paracellular uptake or through mucus penetration with the characteristics of the nanoparticle often dictating the route of entry as well as the biological response induced (Prakash & Matalon, 2014). A summary of these potential entry methods are highlighted in figure 2.1. While inhalation provides an alternative method of pharmaceutical delivery, improving both the treatment and comfort of patients, it can be a double edged sword providing an easy access route for undesirable nanoparticles. A number of studies have been undertaken to explore lung entry of

various nanoparticles and their subsequent distribution around the body. Cerium oxide (CeO_2) nanoparticles have been shown to cause acute toxicity in the lungs following exposure. It was postulated that this could lead to chronic inflammation days after the exposure period, with particles deposited in the lungs as they penetrate through the alveolar wall into the systemic circulation (Srinivas *et al*, 2010; He *et al*, 2010). AgNP exposure in the lung has also been shown to induce cytotoxicity along with other nanometals including ZnO and TiO_2 . The induced toxicity may result in knock-on effects including excessive secretion of certain biological agents causing dysfunction in the cytokine network, thus reducing the efficiency of the respiratory immune system (Liu *et al*, 2013).

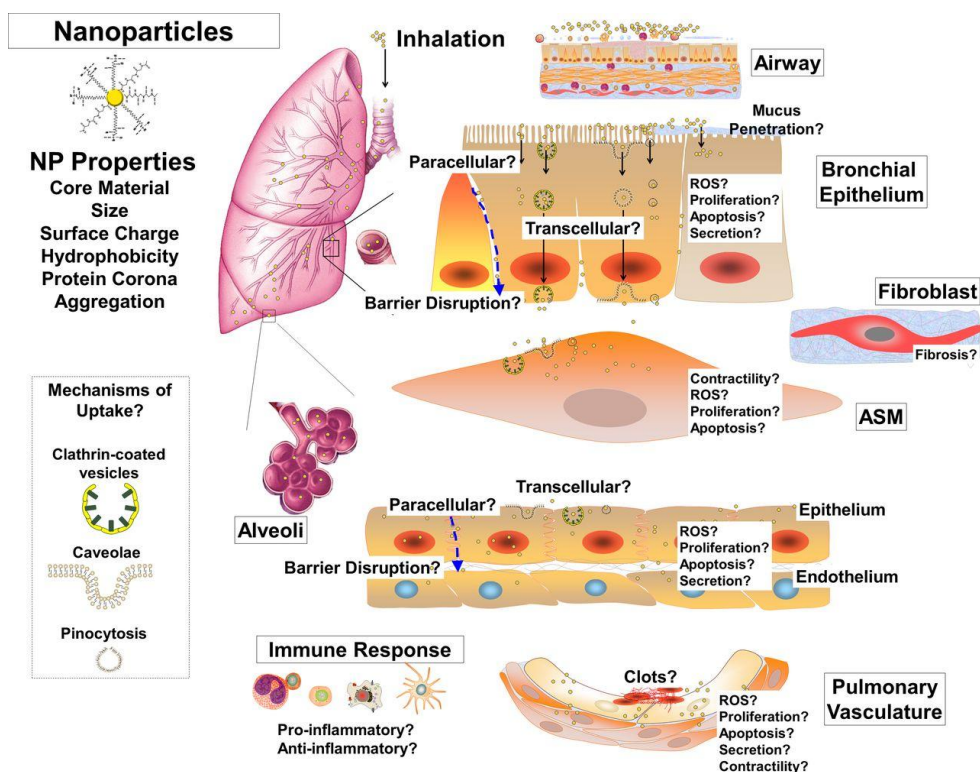


Figure 2.1 Potential routes of absorption of nanoparticles following inhalation and the response induced, dependent on both nanoparticle characteristics and host factors. ASM Airway Smooth Muscle, ROS Reactive Oxygen Species (Prakash & Matalon, 2014)

2.3.3 Oral exposure

Oral exposure is the main exposure route associated with this study. Indeed the probability of nanoparticle exposure via ingestion is high with the advent of nanoparticle use in drug delivery and also their incorporation into food products, to increase the health benefits and assist in adequate nutrient delivery. In terms of drug delivery the oral route is the easiest and most acceptable to patients but due to the various obstacles associated with delivery such as degradation by stomach acid and elimination by the first-pass effect, the stability and final concentration of the drug reaching the systemic circulation comes into question. Nanoparticle systems offer a solution to the problems associated with ingestion of pharmaceuticals. An example of nanoparticle use in oral drug delivery is the use of chitosan nanoparticles. These nanoparticles are biocompatible, non-toxic, have muco-adhesive properties and can open tight junctions in the intestinal tract facilitating their residence time in the gastrointestinal (GI) tract and transport across the gastric epithelium. When loaded with insulin chitosan nanoparticles have been shown to increase the bioavailability and absorption of insulin therefore reducing blood glucose levels in *in vivo* diabetic rat models (Chen *et al*, 2013; Lin *et al*, 2007).

As well as ingestion of nanoparticles due to drug delivery, nanoparticles are used in food products to prolong shelf-life and also to increase the nutritional value of foodstuff by increasing the bioavailability of nutrients.

2.3.3.1 Ingestion

Throughout evolution humans have ingested various nano and microparticles including nutrients such as ferritin, thus demonstrating how the GI tract has adapted to facilitate nanoparticle uptake. This naturally occurring nanoparticle, ingested both in meat and plant based food, appears to be only partially digested in the GI tract and it has been suggested that it may be endocytosed and utilised by epithelial cells in the gut as a source of dietary iron. This process highlights that nanoparticle uptake in the gut can occur and that in some cases it may be beneficial (Powell *et al*, 2010). Once food is partially digested by chewing in the oral cavity, dissolution begins in the stomach due to acidic conditions and the release of various enzymes. Following release from the stomach the food is then exposed to bile salts which will emulsify any fats and

hydrophobic compounds present (Acosta, 2009). Although there are a number of digestion processes nanoparticles will be exposed to, their sub-cellular size can dramatically increase their residence time in the gut. They can achieve this because of the large surface area available to interact with biological entities and their size can contribute to a decreased rate of clearance by intestinal mechanisms. Nanoparticles can efficiently deliver bioactive compounds to various targets in the body because they can pass through fine capillaries, penetrate deep tissues, cross the epithelial lining fenestration and are usually taken up efficiently by cells (Chen *et al*, 2006). Along the inner surface of the small intestine microvilli coat the surface of epithelial cells. These structures are fundamental for nutrient absorption and the layer of mucin covering the surface of the microvilli represents a key component involved in nanoparticle uptake (Acosta, 2009).

The GI tract has been noted as a major area of AgNP deposition coupled with pathological responses. A number of studies have demonstrated damaged microvilli and intestinal glands, abnormal pigmentation and an increase in intestinal goblet cells following oral administration of AgNP in rats (Hadrup & Lam, 2014; Shahare & Yashpal, 2013; Jeong *et al*, 2010; Kim *et al*, 2010). The liver in particular is an important site of nanoparticle deposition following ingestion with reports of high concentrations of AgNP reported in mice 24 hours after exposure. Inhalation also provides a route to the liver. Mucocilliary clearance of nanoparticles can result in entry to the GI tract and deposition in the liver. Accumulation of AgNP can lead to liver toxicity including bile duct hyperplasia and inflammation (Kim *et al*, 2008; Nemmar *et al*, 2002). The liver has also been identified as a one of the main pathways involved in nanoparticle excretion. Hepato-biliary secretion is believed to be a main route of intestinal secretion of nanoparticles with evidence of gold and polystyrene nanoparticle excretion by this route. Accumulation of nanoparticles within bile canaliculi is suggestive of the important role of bile in the intestinal secretion and elimination of nanoparticles in faeces (Zhao *et al*, 2014; Johnston *et al*, 2010; Semmler-Behnke *et al*, 2008).

There are four main pathways by which nanoparticles can cross the gastrointestinal mucosa; transcellular uptake by enterocytes, M-cell uptake, persorption and paracellular uptake. The primary function of enterocytes is to absorb and transport nutrients

systemically, but data has shown that these cells can also take up particulate material (Acosta, 2009).

1) Transcellular uptake

The transcellular transport of particulate material across enterocytes is known as transcytosis. This process begins at the apical cell membrane whereby an endocytic process transports particles through the enterocytes or the M-cells in Peyer's patches and releases them at the basolateral side of the epithelium (Des Rieux *et al*, 2006; Magnuson & Bouwmeester, 2011). There are two methods of transcytosis, one involves the direct diffusion of particles through the enterocyte, this is a passive mechanism and the other requires specific receptors for the transport of particles. This active process uses receptors to capture nanoparticles with specific surface chemistry and internalise them (Acosta, 2009; Des Rieux *et al*, 2006).

2) M-cell uptake

Although enterocytes represent 90-95% of the epithelial cells in the intestine it is through M-cells that is the most common route of uptake for nanoparticles (Powell *et al*, 2010). M-cells are a specialised, differentiated group of epithelial cells located in the Peyer's patches of the lower intestine (Powell *et al*, 2010; Acosta, 2009). They can transport material intact from the lumen to adjacent mononuclear cells and their location is thought to be at important immune inductive sites (Powell *et al*, 2010). M-cells primary function is to sample potential antigens in the intestine and this role provides a mechanism of continued pathogen and antigen surveillance (Powell *et al*, 2010; Acosta, 2009). Although M-cells are more permeable than enterocytes it would seem that these cells are an ideal entry point for nanoparticle absorption, however M-cells represent less than 1% of the total intestine area making delivery of particles more difficult. To overcome this issue there are currently developments to incorporate specific ligands on nanoparticles for M-cell delivery. Due to their antigen monitoring role, M-cells have developed specific receptors to capture various antigens and pathogens coated with glycoproteins and it is this principle that is being explored as a possible method for the targeted delivery of nanoparticles (Acosta, 2009).

3) Paracellular uptake

Another route for nanoparticle uptake in the intestine is through the interstitial space between epithelial cells known as paracellular uptake. However due to the space between the cells ranging from 0.3-1nm, the paracellular route is considered the least effective of the four mechanisms. For the majority of nanoparticles the space between the epithelial cells is just too small to permeate (Acosta, 2009). Although the tight junctions between epithelial cells are extremely efficient at preventing even the smallest nanoparticles to permeate, there are a number of physiological conditions that can affect the integrity of these junctions (Acosta, 2009; Des Rieux *et al*, 2006). Various situations including disease, calcium chelators and epithelial cell metabolism can affect tight junctions allowing the influx of very small nanoparticles (Acosta, 2009). In addition to physiological conditions a number of synthetic peptides can also modulate the influx of nanoparticles through tight junctions. One such peptide is an E-cadherin-derived peptide that expands tight junctions between the endothelium by acting on the aqueous filled pores of the paracellular pathways causing expansion (Magnuson & Bouwmeester, 2011).

4) Persorption

Finally the route of persorption or passage through “gaps” has also been described as a mechanism of nanoparticle uptake. This occurs when enterocytes are shed into the gut lumen from the villous tip leaving a hole in the epithelium through which nanoparticles as well as large particles including starch can be translocated (Acosta, 2009). A summary of all methods of nanoparticle transport across the GI tract is illustrated in figure 2.2.

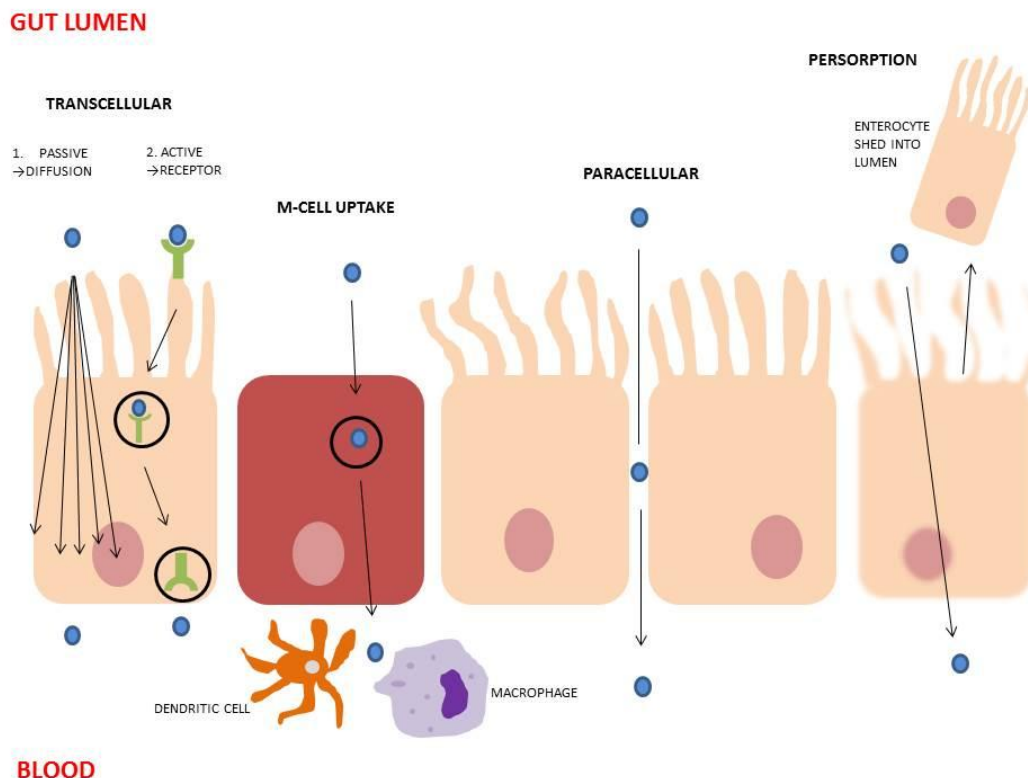


Figure 2.2 Summary diagram of methods of nanoparticle transport in the GI tract

Nanoparticles can be modified to protect from metabolism and local degradation. As a result they can enter intact into the blood and lymphoid circulation from the gastrointestinal epithelium and from here be further distributed to other tissues in the body. Once in the blood nanoparticles can interact with blood components which may influence their distribution and subsequent excretion. There are a number of organs where nanoparticles have been identified including the liver and spleen. It has been revealed that the smaller the nanoparticle the more diverse the distribution within the body with some nanoparticles identified in the bone marrow and brain (Bouwmeester *et al*, 2009).

The method of nanoparticle uptake is influenced by a number of characteristics of the specific nanoparticle. Absorption depends not only on the physicochemical properties of the nanoparticle but on the properties associated with the GI tract. Whether a particle is absorbed or not depends on its initial contact with enterocytes, its ability to access and diffuse through mucus, cellular trafficking and post-translocation events (Medina *et al*, 2007). The transcellular uptake of nanoparticles can be influenced by specific particle

characteristics such as particle size, surface charge, attachment of ligands or coating with surfactants, shape, physical and chemical stability (Magnuson & Bouwmeester, 2011). Different properties of particles can affect not only if they are absorbed but the rate at which they are so. One important factor appears to be charge and studies have shown that anionic nanoparticles can reach the epithelial surface and diffuse across the mucus layer whereas cationic particles become trapped in negatively charged mucus (Magnuson & Bouwmeester, 2011). Size also appears to be a key factor as it has been shown that the smaller the particle diameter the faster particles diffuse through the GI tract and reach the colonic enterocytes (Medina *et al*, 2007).

2.4 GI Tract Defences

As mentioned, oral exposure is the main focus of this report and can include inhalation as well as ingestion. While separate these systems function only millimetres apart and it is possible a certain amount of nanomaterial will enter the lung during ingestion in the oral cavity where both the oesophagus and trachea begin. While this report will include data on nanoparticle toxicity exposure to the lungs it is mainly toxicity due to ingestion and the effects on the GI tract that will be investigated.

On a daily basis the GI tract is exposed to numerous potentially harmful agents, and with a total surface area in the range of 200m^2 , without an adequate defence system harmful pathogens and toxic agents would gain easy access to associated organs and the systemic circulation. While protective mechanisms must be in place for prevention from infiltration of unwanted agents, a balance must be maintained between the defences keeping potentially harmful agents out and normal functions such as exchange of nutrients and ions. As a result the GI tract has a number of defence mechanisms in place that constantly monitor the substances within the intestine. These include commensal gut microflora, the epithelial surface of the intestine, intestinal macrophages and mucosal B cells all of which provide the GI tract with a multi-layered defence system (Schenk & Mueller, 2008).

2.4.1 Commensal Gut Microflora

The microflora that are resident in the gut provide a number of benefits including facilitating the digestion of nutrients such as cellulose. The most important function by far is that these bacteria occupy 'space' within the intestine that would otherwise become occupied by other unwanted disease causing pathogens. While in some instances such as in immunocompromised patients the commensal bacteria may become pathogenic, in the normal healthy individual the relationship is mutually beneficial with microflora providing a barrier defence by maintaining the epithelial villi (Hooper *et al*, 2001; Schenk & Mueller, 2008).

2.4.2 The Epithelial Surface

Within the intestinal epithelium are specialised cells known as goblet cells. Their primary function is to secrete mucus onto the surface of the epithelium which adds to the physical barrier (Mueller & Macpherson, 2006). This complex mix of glycoproteins ensures commensal bacteria remain in the intestinal lumen and do not penetrate the epithelium. Another group of cells called Paneth cells, secrete antibacterial enzymes such as lysozyme upon exposure to invading pathogens, another component preventing access to the intestinal mucosa (Ayabe *et al*, 2000).

2.4.3 Intestinal Macrophages

Bacteria that bypass the aforementioned intestinal barriers and gain access to the mucosa can be phagocytosed and destroyed by intestinal macrophages. These macrophages are also involved in maintaining tissue homeostasis by phagocytosing dead cells and do not display potent inflammatory cytokine production. This feature prevents an overstimulation of the immune system to commensal bacteria if they gain access to the mucosa (Sansonetti & Braesco, 2001; Smith *et al*, 2001; Schenk & Mueller, 2008).

2.4.4 Mucosal B cell System

B cells are antibody producing lymphocytes and mucosal B cells provide an active defence by terminally differentiating into plasma cells that secrete only IgA antibody.

Once secreted this IgA enters the intestinal lumen and can bind to invading pathogens reducing their motility and adhesive properties (Kallies *et al*, 2004; Kaetzel *et al*, 1991; Schenk & Mueller, 2008).

2.5 Lung Defences

The lung serves as a massive surface area between the external environment and the host and as such requires a multilayered defence system to protect from invading pathogens that are constantly inhaled. There are two types of defence mechanism found in the lung, the initial mechanical response mechanism and the innate immune response. The first line of defence involves non-specific receptors that line the lung involved in mucociliary clearance, cough reflex and the ciliary beat removing invading pathogens. While this initial line of defence is important it has now been discovered that a more sophisticated innate immune defence is present providing a key role in removal of potentially harmful pathogens. Pulmonary epithelial cells produce a variety of small molecules that are involved in innate immunity. Two of these secreted components SP-A and SP-D have been shown to play an important role in host defence (Zaas & Schwartz, 2005; LeVine & Whitsett, 2001).

2.5.1 Surfactant protein A (SP-A)

SP-A is a surfactant associated protein that is expressed by alveolar type II cells and binds dipalmitoylphosphatidylcholine (DPPC). Its key role in host defence is enhancing microbial phagocytosis by macrophages. It acts as an opsonin that stimulates uptake of a variety of pathogens by alveolar macrophages. As well as enhanced macrophage uptake, SP-A can also induce calcium dependent neutrophil uptake of certain pathogens including *E. coli* (LeVine & Whitsett, 2001).

2.5.2 Surfactant protein D (SP-D)

SP-D is expressed in pulmonary tissue but is also found in other tissues in the body. Like SP-A, it is expressed by alveolar type II cells and binds phosphatidylinositol in pulmonary surfactant. SP-D has been shown to bind and aggregate bacteria enhancing their phagocytosis by alveolar macrophages. In addition to bacteria, SP-D can also bind

and aggregate certain viral and fungal surfaces including influenza A and *Aspergillus* (LeVine & Whitsett, 2001).

2.6 Role of Biofluids

A biofluid is any fluid originating from within the body that can be excreted, secreted, obtained by a needle or can develop due to a pathological process and ranges from blood to bile and breast milk to cyst fluid (Medicinenet). There are numerous biofluids in the body including saliva, blood, urine, stomach acid and bile to name a few. Each biological fluid has its own important individual role within the body. Some of those functions include:

- Blood: oxygen transport
- Saliva: breakdown of starch by amylase and aids in chewing
- Urine: excretion of waste metabolites and excess water
- Stomach Acid: aids in further digestion and breakdown of food via enzymes
- Bile: hydrolyses fat

In addition to the vital physiological role biofluids perform within the body, they have also been shown to aid in nanoparticle processing including isolating particles from larger agglomerates, particle coating and modification of surface chemistry (Herzog *et al*, 2009). In this study components of two major biofluids found in the body will be investigated, ursodeoxycholic acid (UDCA), cholic acid (CA) and deoxycholic acid (DCA) that form part of total bile and dipalmitoylphosphatidylcholine (DPPC) a component of lung surfactant.

2.6.1 Dipalmitoylphosphatidylcholine (DPPC)

Pulmonary surfactant is composed of a variety of complex phospholipids, lipids and surfactant specific proteins. DPPC is the most abundant phospholipid, comprising 70-80% of total surfactant. Pulmonary surfactant is synthesized by type II pneumocytes and any deficiency of this fluid can result in serious respiratory disorders such as Acute Respiratory Distress Syndrome (ARDS). The overall function is to reduce surface tension at the liquid-air interface at the bronchoalveolar surface (Kumar & Bohidar,

2010; Serrano & Perez-Gil, 2006). During inspiration, surfactant lowers surface tension to near equilibrium, minimizing the work of breathing. During exhalation, surface tension values are reduced, stabilizing the lungs at low air volume and preventing pulmonary oedema (Bakshi *et al*, 2008). In order to perform these functions all of the components of lung surfactant are required to work in synergy.

2.6.2 Bile Acids

Bile acids are steroidal detergents synthesized from cholesterol by hepatocytes which drain into the bile duct, hepatic duct and finally into the gall bladder for storage. During digestion and in response to several enzymes, bile is released into the duodenum where its principle function is lipid digestion. On average 0.4-0.8L of bile is produced each day with over 95% reabsorbed in the intestine and returned to the gall bladder. Bile acids themselves are amphiphatic molecules with an unusual structure in that they do not have a well-defined head and tail group and exist as flat rigid molecules. They can be divided into two groups (1) primary bile acids, synthesized in the liver from cholesterol and (2) secondary bile acids, formed following bacterial hydrolysis of the amide backbone of conjugated bile acids in the large intestine. Bile acids provide a crucial function in digestion by solubilising and transporting fat soluble nutrients across the intestinal mucosa. Bile acids adsorb onto the surface of digested lipids and remove any bound proteins from the surface allowing easy lipid lipolysis by lipid enzymes (Kumar & Bohidar, 2010; Maldonado-Valderrama *et al*, 2011; Holm *et al*, 2013). For this study three bile acids were investigated;

1. Ursodeoxycholic Acid (UDCA)
2. Cholic Acid (CA)
3. Deoxycholic Acid (DCA)

1. Ursodeoxycholic Acid (UDCA)

UDCA is a secondary bile acid produced from primary bile acids metabolized by gut bacteria, comprising 3% of total bile acids (Perez & Briz, 2009). Despite its low abundance it is an extremely interesting bile acid as it has been shown to have direct antioxidant properties against hydroxyl radicals and can also prevent the retention of

certain toxic hydrophobic bile acids. UDCA also has a number of therapeutic applications including dissolving gall stones and as an alternative therapy for hepatitis C patients in place of interferon therapy (Arisawa *et al*, 2009; Lapeena *et al*, 2002).

2. Cholic Acid (CA)

Cholic acid, along with chenodeoxycholic acid (CDCA), is one of the major primary bile acids in the liver and is synthesized from cholesterol. Cholic acid constitutes about 30-40% of bile acids and together with CDCA makes up almost 80% of the bile acid component of total bile (Debruyne *et al*, 2001). Formation of these bile acids is important in cholesterol homeostasis. The specific enzyme for cholic acid synthesis is 12 α -hydroxylase which is important in determining the ratio of cholic acid to CDCA. Tight regulation of this synthesis is required as it is believed that a change in the ratio of these two bile acids may result in gallstone formation due to a reduction in the conversion of cholesterol to bile acids (Del Castillo-Olivares & Gil, 2000).

3. Deoxycholic Acid (DCA)

Deoxycholic acid (DCA) is a secondary bile acid resulting from the anaerobic dehydroxylation of unabsorbed cholic acid by bacteria residing in the gut. This process occurs in the colon on any cholic acid that has not been absorbed in the ileum. DCA constitutes roughly 10-40% of bile acids in total bile (Marcus & Heaton, 1988). DCA has been noted to promote colonic epithelium proliferation and this proliferation is related to serum DCA levels (Deschner *et al*, 1981; Ochsenkuhn *et al*, 1999). This bile acid along with others including cholic acid has been implicated in colorectal malignancies. It has been observed that bile acids can stimulate the growth of malignant cells and promote their invasion. Certain conjugated bile acids have also been implicated in breast cysts as elevated levels of these bile acids were observed in cyst fluid. This may indicate a potential role of glycine conjugated bile acids including cholic acid, CDCA and DCA in breast malignancies. However other bile acids are also being exploited as therapies for cancer treatment. UDCA is the most promising with investigations into a targeted therapy for colorectal cancer (Debruyne *et al*, 2001).

2.7 The Immune System

An intact and functioning immune system is vital in the protection against harmful external stimuli. Inflammation is the process by which cells of both the innate and adaptive immune system are activated in order to remove an invading agent and repair any damage caused. The controlled and rapid initiation and effective resolution of inflammation is vital to maintain normal homeostasis.

The innate immune response is the first line of active defence and comprises a number of phagocytic cells, including macrophages, neutrophils, and the complement system. Monocytes in particular are the primary cell type involved in the initial immune response to external stimuli, recognising distinct patterns (PAMPs) on the surface of pathogens such as lipopolysaccharide (LPS) via receptors on the cell surface, toll-like receptors (TLRs) (Palm & Medzhitov, 2009; Beutler, 2009; Oberbarnscheidt *et al*, 2011). Recognition of these patterns is vital in the detection of an infection but also in determining the type of infection. The response to these PAMPs involves the release of a variety of pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF- α). Monocytes are one of the main mediators of the initial inflammatory response with an important role in phagocytosis of foreign material at entry sites (Beutler, 2009; Oberbarnscheidt *et al*, 2011). Their production of inflammatory mediators including IL-1, IL-6 and TNF- α , are vital to the initial immune response. These cytokines have profound effects on associated cells and the local microenvironment such as leukocyte migration, endothelial adhesion increased neutrophil activation and antibody secretion all contributing to the inflammatory response (Rehman *et al*, 2012; Medzhitov & Janeway Jr, 2000; Kawai & Akira, 2007). IL-1 β is of particular interest due to its central role in the inflammatory process. IL-1 β exerts a number of effects including influencing immune cells such as macrophages and endothelial cells and also producing systemic effects. Cleavage to its mature form is essential for directing the host response to injury or infection.

2.7.1 Interleukin-1 (IL-1)

The IL-1 family comprises eleven members of which most are pro-inflammatory but some can have anti-inflammatory functions. IL-18 and IL-1 β are the most fully characterised and have an unusual property in that they are initially expressed as

inactive pre-cursors that require additional processing to become biologically active (Monteleone *et al*, 2015; Chen & Schroder, 2013). IL-1 β in particular is the focus of many studies due to its highly active state and its secretion from immune cells. Its functions range from recruitment of immune cells to induction of fever (Dinarello, 1996). It has been shown that IL-1 β is involved in certain pathologies, is not required for normal homeostasis and that a deficiency in this cytokine can have a protective effect (Joosten *et al*, 2013; Dinarello, 2011).

Monocytes are the main source of IL-1 which is pivotal in co-ordinating the early immune response. This cytokine is the most potent fever inducing molecule and as such is tightly regulated. Over-expression or dysregulation of IL-1 can cause auto inflammatory disorders and contributes to the progression of septic shock (Goldback-Mansky & Kastner, 2009; Dinarello, 2009; Dinarello, 1996). IL-1 has been implicated in a number of acute and chronic inflammatory disorders and as a result IL-1 β has been isolated as a therapeutic target for the treatment of certain disorders including rheumatoid arthritis and autoinflammatory syndromes that are characterized by local and systemic inflammation, recurrent fevers and fatigue (Dinarello, 2011; Dinarello, 2013).

2.7.2 Interleukin-6 (IL-6)

IL-6 is a well-defined multi-functional cytokine involved in the proliferation and differentiation of cells involved in inflammation. IL-6 activation occurs through binding to its receptor which can be either membrane bound or soluble (Scheller *et al*, 2014). IL-6 has demonstrated a variety of functions in addition to its contribution to the inflammatory process, including liver regeneration, bone remodelling and metabolism (Spooren *et al*, 2011). It has also been shown to have anti-inflammatory properties with an important role in regulating the levels of other pro-inflammatory cytokines suggesting a role in the prevention of dysregulation of certain cytokines (Xing *et al*, 1998; Spooren *et al*, 2011). The opposing effects exhibited by IL-6 are attributed to the two receptor types with the soluble receptor responsible for the pro-inflammatory effects and binding to the membrane bound form accounts for the anti-inflammatory effects (Wolf *et al*, 2014).

IL-6 along with other cytokines including TNF- α , can stimulate the release of chemokines to attract neutrophils to the site of inflammation and can contribute to the overall systemic reaction in response to inflammation (Scheller *et al*, 2011; Gruys *et al*, 2005). IL-6 induction forms part of the normal initial inflammatory response resulting from a disruption to the body's normal homeostasis but its overexpression has been implicated in a number of pathologies. These can range from autoimmune diseases including thyroiditis, rheumatoid arthritis to cancer (Feghali & Wright, 1997). Expression of this cytokine has been shown to be a contributing factor in the progression of cancers such as colitis associated colon cancer and Kaposi sarcoma. An increase in levels of circulating IL-6 has also been implicated in the development of Hodgkin's lymphoma (Lin & Karin, 2007; Scheller *et al*, 2011).

2.7.3 Tumour Necrosis Factor Alpha (TNF- α)

TNF- α is a multi-functional molecule which plays a vital role in acute inflammation and has been described as a very potent cytokine. Along with other pro-inflammatory cytokines it is important not only in the initiation, but also in the maintenance of inflammation (Nathan, 2002). Released by a number of cells upon stimulation, mainly macrophages, TNF- α can mediate a number of responses directly such as fever and tissue damage, release of acute phase proteins from the liver in addition to cell proliferation, differentiation and apoptosis (Nathan, 2002; Hehlhans & Pfeffer, 2005). This cytokine can also exert its effects indirectly by downstream signalling leading to the activation of other inflammatory factors, such as IL-6, augmenting the inflammatory response by activation of the cytokine cascade as well as acting as a key factor in monocyte adhesion to the endothelium by up-regulation of several adhesion molecules including ICAM-1 (Feghali & Wright, 1997; Warren, 1990; Ende *et al*, 2014).

In addition to the beneficial role of TNF- α in the inflammatory process and homeostasis, this cytokine can produce damaging effects to the host. TNF- α has systemic endotoxic activity that when produced in large quantities, contributes to fever and shock (Feghali & Wright, 1997; Beutler & Caerami, 1988). Dysregulation can lead to a chronic inflammatory state and can result in a number of pathological conditions. Some of the conditions TNF- α has been implicated in include rheumatoid arthritis and inflammatory bowel conditions (Hehlhans & Pfeffer, 2005). As a result, blocking of

TNF- α has been and continues to be investigated as a potential therapy for certain inflammatory disorders (Wong *et al*, 2008).

2.7.4 The Inflammasome

Induction of IL-1 β is particularly interesting as maturation to its fully functioning form is tightly regulated by the inflammasome. The inflammasome is a complex of multiple proteins found in the cytoplasm and requires a number of danger signals such as tissue stress in order to trigger inflammatory caspases. Initial signalling in the innate immune response via TLRs leads to the transcription of pro-IL-1 β but for complete activation a second signal detected by intracellular sensor molecules is required to activate the inflammasome, resulting in caspase-1 mediated cleavage to the mature form. The “sensing” of both signals in a two-step activation process is essential in controlling the cleavage of cytokines like IL-1 β and IL-18 to produce an effective and controlled inflammatory response against infective agents (Martinon *et al*, 2002; Latz *et al*, 2013; Terlizzi *et al*, 2014; Schroder & Tschopp, 2010; Gross *et al*, 2011).

It is now widely accepted that in order to produce the mature active form of IL-1 β , gene expression of this cytokine alone is not sufficient and requires involvement of the inflammasome. The inflammasome is an important platform allowing for caspase-1 mediated maturation of IL-1 β . While a variety of stimuli can induce gene expression of IL-1 β , in order to exert its immunological effects the pro-form must be cleaved by caspase-1 in order to produce the mature molecule. The most prominently investigated inflammasome is NLRP3 as it is the most fully characterised, diverse and clinically significant of all inflammasomes and can be activated by exposure to pathogens and other environmental irritants (Schroder & Tschopp, 2010). Caspase-1 activity which is vital for pro-IL-1 β cleavage to the bioactive form is tightly regulated by the NLRP3 inflammasome (Latz *et al*, 2013; Schroder & Tschopp, 2010). In addition to proteolytic processing of pro-IL-1 β , caspase-1 is also required for a form of cell death known as pyroptosis. This results in rapid pore formation in the plasma membrane of cells leading to osmotic cell lysis (Satoh *et al*, 2013). Cleavage to the bioactive form is essential in order for IL-1 β to perform its immunological effects which range from co-ordinating early immune response through cell activation to systemic responses such as regulation of appetite, temperature and sleep (Dinarello, 2009; Chen *et al*, 2011; Kasza, 2013;

Yang *et al*, 2012). Inflammasome activation can also play a role in adaptive immunity as cytokines resulting from its activation are involved in further signalling including differentiation of T cells into the various subtypes (Chen *et al*, 2011).

2.8 Nanotoxicity

Toxicity can be defined as the degree to which a substance can harm living organisms. The area of toxicology studies the interaction and effects of such substances on living organisms (Timbrell, 1998). As the interaction between living organisms, the environment and various nanoparticles becomes more common it is now important to focus not only on the benefits provided by these nanoparticles but to also establish any risk associated with exposure. Based on the basic definitions of toxicity, nanotoxicology can be described as the study of the toxic effects of nanoparticles on living organisms and the environment (Donaldson *et al*, 2004; Paur *et al*, 2011; Arora *et al*, 2012). The area of nanotoxicology aims to provide answers to some of the concerns raised about the safety of nanoparticle exposure and to provide clear, conclusive data regarding the relationship between nanoparticles including their concentration metrics and physicochemical characteristics, and biological systems. The mechanism by which nanoparticles mediate their toxicity is also an important factor to be considered (Paur *et al*, 2011; Suh *et al*, 2009; Lewinski *et al*, 2008; Donaldson *et al*, 2004). AgNP are becoming of particular interest in nanotoxicology as although they provide a number of benefits including antibacterial properties it is now apparent that on an ultra-small scale, they can induce toxicity following internalisation and migrate to various organs around the body (Sung *et al*, 2008; DeVasConcellos *et al*, 2012; Antony *et al*, 2015).

2.8.1 Nanoparticle Toxicity in the G. I Tract

In this report the focus is on entry and resulting toxicity following oral exposure to AgNP with a focus on the GI tract, including the lungs, and subsequent entry into the blood stream. Next to inhalation ingestion of nanoparticles is probably one of the most common routes of exposure. While toxicity in the lungs following inhalation of AgNP has been well documented, toxicity brought about by ingestion and the subsequent effects induced are still not fully understood (Gaillet & Rouanet, 2015). It has been well documented that prolonged exposure via ingestion to silver results in systemic argyria

but a full cytotoxic profile of AgNP effects on the GI tract is still required (Gulbranson *et al*, 2000; White *et al*, 2003).

With a total surface area in the range of 200m² (although this size has recently been disputed indicating a smaller surface area in the range of roughly 32m²), required for optimum nutrient exchange and absorption, the GI tract provides one of the largest surfaces within the body that any internalised nanoparticle will come into contact with (Powell *et al*, 2010; Helander & Fändriks, 2014). As discussed previously the GI tract has been exposed to naturally occurring nanoparticles throughout evolution and has the capacity to absorb and internalize materials on the nanoscale via a number of uptake mechanisms (Powell *et al*, 2010; Acosta, 2009; Des Rieux *et al*, 2006). This theory can also be applied to manmade or engineered nanoparticles. Following absorption it is possible for AgNP to be transported to various organs in the body including the liver and eventually enter the circulation (Shahare *et al*, 2013; Bergin & Witzmann, 2013). The liver in particular operates closely with the GI tract during digestion and is important in the detoxification of various harmful substances. A number of studies have reported liver toxicity in response to AgNP exposure and also highlighted it as a major site of particle accumulation (Hussain *et al*, 2005; Takenaka *et al*, 2001; Hadrup & Lam, 2014; Kim *et al*, 2008; Sardari *et al*, 2012). When evaluating the toxicity of AgNP on the GI tract it must be taken into account the variety of substances the particles will have contact with. These substances range from the commensal gut microflora that reside in the intestine to the various biological fluids involved in the digestive process such as bile and stomach acid, to partially digested food, all which may influence the particles physicochemical profile and resulting biological response (Walczyk *et al*, 2010; Mahmoudi *et al*, 2011).

2.8.2 Nanoparticle Toxicity and the Immune System

The immune system is a vital component of the body's defence against foreign elements such as pathogens and particulates (Chaplin, 2010). Understanding this complex and sophisticated system is important when investigating nanoparticle toxicity in order to develop a complete profile of the interaction of nanoparticles with immune cells and the resulting response induced (Zolnik *et al*, 2010; Farrera & Fadeel, 2015). Several studies have focused on investigating the immune-modulatory effects of nanoparticles both in

pristine form and following their interaction with biological proteins leading to alterations in the responses induced. These studies have looked at two aspects of nanoparticle interaction with the immune system: beneficial and harmful (Paino & Zucolotto, 2015; Barkhordari *et al*, 2014; Tian *et al*, 2007; Xu *et al*, 2013).

Nanoparticles have been shown to initiate inflammation and a subsequent immune response. For instance carbon nanotubes (CNTs) can affect the phagocytic function of macrophages leading to an impaired ability to clear an invading pathogen. CNTs may also impair dendritic cell function, which are vital in stimulating T and B cell responses thus linking the innate and adaptive responses. This impairment results in immunosuppression reducing the body's defence against infection (Dumortier, 2013; Laverney *et al*, 2013). AgNP have been noted as potent triggers of an inflammatory response resulting from interaction with immune cells including macrophages producing cytokine release and immune cell recruitment with ROS generation often acting as a contributing factor (Kim & Choi, 2012; Park *et al*, 2011; Lim *et al*, 2012; Martinez-Gutierrez *et al*, 2012). One of these AgNP mediated responses involves IL-1 β release, a potent cytokine involved in innate immunity which is involved in widespread systemic effects through activation of the inflammasome (Simard *et al*, 2015; Yang *et al*, 2012). The involvement of AgNP in its release highlights the immunological significance of these nanoparticles and may have pathological consequences or contribute to already existing diseases (Birrell & Eltom, 2011; Yang *et al*, 2012).

As previously mentioned nanoparticle immune system interactions can be manipulated to provide desirable immune-modulatory effects. An investigation into engineered nanoparticles has highlighted their potential use as antigen-presenting cells and carriers of immunostimulatory molecules for vaccination purposes (Zolnik *et al*, 2010; Farrera & Fadeel, 2015). Nanomaterials are also being investigated as potential adjuvants. Adjuvants are important components of vaccines enhancing immunogenicity of a particular antigen thus increasing a protective immune response. AgNP have been targeted as a potential adjuvant with a study demonstrating significant adjuvant effects when tested with bovine serum albumin (BSA) and ovalbumin (OVA) in mice. Increased levels of specific antibodies along with leukocyte recruitment highlighted the potential biomedical application of AgNP as an adjuvant (Xu *et al*, 2013). While these initial findings look promising AgNP use as an adjuvant is a long way off considering their toxicity.

The two sided argument for nanoparticle interaction with the immune system leaves a lot of questions still unanswered as to the variety of responses induced by exposure, the role of physicochemical transformation upon entry has on responses induced and also where to find a balance between the desirable and harmful effects of nanoparticles both natural and engineered on the immune system.

2.9 Summary

Nanotechnology has a wide variety of applications in consumer products and as a result the potential contact with living systems cannot be avoided. This has resulted in concerns being raised about the potential risk nanoparticle exposure has on the health of humans and also its environmental impact. AgNP in particular have been highly regarded for their benefits but it is only now coming to light the health risks they pose. As such a more in depth analysis of their toxic potential is required including looking at their interaction with various components of biological systems and the resulting responses produced following oral exposure. This includes assessing interactions with components of biofluids such as bile and lung surfactant to establish if this can influence the biological response provoked by AgNP as well as the consequences of AgNP entry into the circulation. From the literature reviewed and the evidence provided on the toxicity of AgNP exposure, this study aims to investigate the modification of AgNP physicochemical characteristics and toxicity by biofluid components. In addition an investigation into the induction of an innate immune response following particle absorption into the blood will be undertaken. Hopefully this study can provide a more realistic and detailed investigation into the toxicological effects of AgNP and contribute to the on-going development of a complete risk assessment detailing the potential risk posed by exposure to AgNP.

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Chapter 3

General Experimental

3.1 Introduction

This chapter will introduce and outline the various techniques used in characterising and determining the cytotoxic effect of AgNP. Full characterisation of nanoparticles is crucial in assessing the size, shape, stability and other properties of particles prior to cytotoxicity testing. It is these properties which will ultimately affect how AgNP will interact with cells and other biological entities upon entering the body and determine their cytotoxic profile. The AgNP in question are PVP coated particles purchased from Sigma-Aldrich, Dublin (Catalogue No: 758329). Together with the physicochemical characterisation, the methods used for cytotoxic, inflammatory and immunological evaluation will also be discussed in this chapter.

3.2 Test materials and reagents

Polyvinylpyrrolidone (PVP) coated silver (Ag) nanopowder of < 100nm Catalogue No: 758329, was purchased from Sigma-Aldrich Ltd (Dublin, Ireland). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Catalogue No: M5655 and 2', 7'- dichlorofluorescein diacetate (DCFH-DA) Catalogue No: D6883 as well as cell culture media, supplements, trypsin solution, lipopolysaccharide (catalogue No: L4391), polymyxin B (catalogue No: P0972), Tri-reagent (catalogue No: T9424), 1-Bromo-3chloropropane (catalogue No: B9673), 2-propanol (Catalogue No: I9516), ethanol (Catalogue No: E7023), PCR primers, adenosine 5'-triphosphate disodium salt hydrate (ATP) (Catalogue No: A6419) and Ac-YVAD-CMK an inhibitor of the inflammasome were all purchased from Sigma-Aldrich Ltd (Ireland). Alamar blue (AB) was purchased from Biosciences (Dublin, Ireland). UDCA (Catalogue No: U5127), CA (Catalogue No: C1129), DCA (Catalogue No: D2510) and DPPC were purchased from Sigma-Aldrich (Dublin, Ireland). Reverse transcription qScript™ kit (Catalogue No: 95047) was purchased from Quanta Bioscience (Maryland, USA). Two step reverse transcription of DNA was performed using LightCycler® 480 SYBR Green I Master kit (Catalogue No: 04 887 352 001) purchased from Roche (Sussex, UK).

3.3 Characterisation of nanoparticles

Prior to the cytotoxicity testing, pristine AgNP and AgNP in various dispersions were characterised. The suspensions of AgNP were prepared in deionised water (dH₂O), DMEM and RPMI media using a bath-sonicator for 20 minutes (Degussa-Ney ULTRASONIK 57X 50/60 Hz, California, USA) prior to size and zeta potential analysis.

3.3.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) generates a number of signals at the surface of specimens using a focused beam of high-energy electrons. Signals received from the electron-sample interaction reveal information regarding the external morphology, chemical composition, crystalline structure and orientation of the sample. A 2-dimensional image of the sample is generated by data collected over a selected area of the sample surface. Using conventional SEM techniques an area of 1cm to 5 microns can be imaged.

Scanning electron microscopy was employed to estimate nanoparticle size from the images produced by this technique. A Hitachi SU 6600 FESEM instrument was used to obtain images of the AgNP. First the SEM was calibrated with Au on Carbon standard provided by Agar Scientific (Essex, UK). Samples were prepared by dispersing particles in ethanol by sonication 750 watts Ultrasonic Processor tip (Branson Ultrasonics, Ultrasonic processor VCX-750W) at 40% amplitude for a total of 45 seconds. Samples were spin coated onto pure silicon wafers which had been thoroughly cleaned by sonication in acetone for 30 minutes followed by boiling in propanol for 30 minutes. Silicon wafers were then left to air dry in a dust free environment and the nanoparticle sample was then spin coated onto wafers 24 hours prior to measurement.

3.3.2 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a technique employed for measuring the hydrodynamic radius of particles. Particles are illuminated by a laser which scatters light due to Brownian motion. Smaller particles are moved further and more rapidly producing greater fluctuations of light. The intensity of this light scatter is fluctuated at a rate dependant on the size of a particle. Analysis of these fluctuations or scatter pattern

yields information on the hydrodynamic radius of the particle using the Stokes-Einstein equation (equation 3.1):

$$D = \frac{kT}{6\pi\eta R}$$

Equation 3.1 Stokes-Einstein equation

D is the diffusion constant

k_B is Boltzmann's constant

T is absolute temperature

η is the viscosity of the medium

r is the radius of a spherical particle

DLS and zeta potential measurements were performed with the aid of a Malvern ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK) operating with version 5.10 of the systems Dispersion Technology Software (DTS Nano). For size measurement DTS0012 disposable sizing cuvettes were used. The samples were equilibrated at 25°C for 2 minutes before each measurement.

3.3.3 Zeta Potential Analysis

Zeta potential is a measure of particle stability. The liquid layer surrounding a particle consists of two parts an inner region where ions are tightly bound and an outer region where they are less firmly bound. Within the outer layer is a boundary known as the slipping plane where ions and particles form a stable entity and the potential at this boundary is the zeta potential.

Zeta potential measurements were performed with the aid of a Malvern ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK) operating with version 5.10 of the systems Dispersion Technology Software (DTS Nano). For zeta potential analysis, DTS1060T clear disposable zeta cells were used and measurements were performed

with the automatic model setting, using a voltage of five to minimize artefacts and charring of media proteins during analysis.

3.3.4 BET Analysis

The specific surface area of AgNP was established with a Micrometrics GEMINI BET. BET sample holders were filled with a known mass of powdered nanoparticles and measured. The sample was degassed for two hours at room temperature with nitrogen gas prior to analysis. Nitrogen gas was used as the absorptive gas and a multipoint method was used in the estimation of specific surface area.

3.4 Cell culture

HepG-2 cells (ATCC: HB-8065) an immortalized hepatocellular carcinoma cell line, Hep2 (ATCC: CCL-23) a cell line of laryngeal origin, A549 (ATCC: CCL-185) an immortalized carcinogenic alveolar cell line and the human monocyte cell line THP-1 (ATCC: TIB202) were employed this study. HepG-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2mM L-glutamine, sodium pyruvate and supplemented with 10% foetal bovine serum (FBS) at 37°C in humidified 5% CO₂. Hep2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 45 IU/ml penicillin and 45 IU/ml streptomycin at 37°C in humidified 5% CO₂. A549 cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and 45 IU/ml penicillin and 45 IU/ml streptomycin at 37°C in humidified 5% CO₂. The THP-1 cell line was cultured in RPMI 1640 (Sigma-Aldrich, Ireland) supplemented with 12.5% foetal bovine serum and 2mM L-glutamine. Cells were incubated at 37°C in humidified 5% CO₂. All cell culture media including FBS other reagents and supplements are from Gibco purchased through Biosciences (Dublin, Ireland).

3.5 Cytotoxic evaluation

The AB and MTT assays were performed for assessment of cytotoxicity of AgNP to the cell lines. The test used a range of eight concentrations of AgNP (3.91-500µg/ml) in

which effects were likely to occur, this in turn allowed inhibitory concentration (IC_{50}) to be calculated. In all cases results were compared to an unexposed control (cells in culture media only), eliminating any dependence of the cell line exposures on well type, seeding efficiency and numbers, exposure times and volumes. A stock suspension of AgNP was prepared aseptically from which different concentrations of nanoparticles were prepared in the respective HepG-2, Hep2, A549 and THP-1 cell media followed by sonication (Branson Ultrasonics, Ultra sonic processor VCX-750W, maximum amplification 40%). As a positive control 10% DMSO was prepared in the respective media. For cytotoxic analysis of nanoparticles in the presence of biofluid components, a range finding experiment was performed to determine concentration of interest for the definitive experiment. A stock solution of this test concentration was prepared for each biofluid component, UDCA, CA and DCA, and the surfactant DPPC in the appropriate cell media. A concentration finding experiment for each bile acid was performed in the respective cell lines to determine a working concentration for cytotoxic studies. The working concentration was noted to have no cytotoxic effect when exposed to cells alone. Based on the concentration finding experiments and previous studies, concentrations of 50 μ M, 1mM and 0.125mM were prepared for UDCA, CA and DCA respectively and a concentration of 2.2×10^{-7} M UDCA was prepared for Hep2 cell line (Arisawa *et al*, 2009). Using this solution of each biofluid component and DPPC, a stock dispersion of AgNP was prepared aseptically from which a range of nanoparticle concentrations was prepared. For cytotoxic analysis of nanoparticles in the presence of DPPC a concentration of 0.25 μ g/ml was chosen based on previous studies (Herzog *et al*, 2009). A stock solution of this test concentration (0.25 μ g/ml) was prepared for DPPC in RPMI-1640. For all assays and exposure scenarios fresh nanoparticle suspensions were prepared for each experiment.

3.5.1 Alamar Blue assay

The alamar blue assay is a cell viability assay that measures the proliferation of cells. Growing cells maintain a reducing environment in their cytosol that causes the REDOX indicator contained in the AB dye to be converted from the non-fluorescent resazurin, to the fluorescent resorufin dye. The fluorescence is measured and relates to the quantity of proliferating cells present.

For the AB assay, cells were seeded in 96 well microtitre plates (Nunc, Denmark) at a density of 1×10^5 , 5×10^4 , 4×10^4 and 3×10^4 cells per ml for 24, 48, 72 and 96 hour exposures respectively in 100 μ l of cell culture media containing 10% FBS. At least three independent experiments were conducted with six replicate wells employed per concentration per plate in each independent experiment. After 24 hours of cell attachment, plates were washed with 100 μ l per well PBS and treated with increasing concentrations of AgNP prepared in media for 24, 48, 72 and 96 hours. All incubations were performed at 37°C in a 5% CO₂ humidified incubator. The assay was performed according to manufacturer's instructions. Briefly, control media and test exposures were removed, cells were rinsed with 100 μ l PBS and 100 μ l of AB solution (5% [v/v] solution of AB) prepared in fresh media with no added supplements were added to each well. After 3 hour incubation AB fluorescence was measured at excitation and emission wavelength of 531nm and 595nm respectively in TECAN GENios (Grodig, Austria). Wells containing AB solution and media only were used as blanks. For this assay the mean fluorescence units for six replicate cultures were calculated for each exposure treatment. Acellular studies were performed with test particles and the AB dye to confirm no interference of the particle with dye conversion (Gupta Mukherjee *et al*, 2012).

3.5.2 MTT assay

The MTT assay is used to measure the mitochondrial integrity of cells. Viable cells depend on an intact mitochondrial respiratory chain. When cells are alive the yellow tetrazolium salt (MTT) is cleaved to a purple formazan dye by succinate dehydrogenase a component of the mitochondrial respiratory chain. Cells are then solubilised with an organic solvent and the absorbance is measured.

As with the AB assay a parallel set of three plates were set up for the MTT assay, seeded and exposed as previously described. After the same exposure times as AB assay, 24, 48, 72 and 96 hours, test medium was removed. Cells were washed with 100 μ l PBS and 100 μ l of freshly prepared MTT solution (5mg/ml MTT in media {without supplements}) was added to each well. After 3 hour incubation the solution was removed, cells were rinsed 100 μ l PBS and 100 μ l MTT fixative (DMSO) was added to each well. Plates were shaken at 240rpm for 10 minutes. Following this step the

supernatant was removed and transferred to a new 96 well plate for analysis as it has been observed that sedimentation of AgNP on the bottom of wells interferes with absorbance readings, producing higher values. Absorbance was read at 595nm in TECAN GENios (Grodig, Austria). Acellular studies were performed with test particles and the MTT dye to confirm no interference of the particle with dye reduction (Gupta Mukherjee *et al*, 2012).

3.5.3 Reactive Oxygen Species (ROS) studies

The ROS assay uses a cell permeable fluorogenic probe, dichlorodihydrofluorescein diacetate (DCFH-DA) to detect intracellular reactive oxygen species. DCFH-DA is diffused into cells and deacetylated by cellular esterases to the non-fluorescent DCFH. DCFH is then rapidly oxidised to the fluorescent DCF by ROS. The fluorescence is measured and is directly proportional to the levels of ROS in cells.

Intracellular oxidative stress was quantified using a 2', 7'-dichlorofluorescein diacetate (DCFH-DA) plate assay to detect intracellular hydroperoxides and probe for a wide range of ROS. Confluent cells were trypsinized and seeded at a density of 1×10^5 cells per ml prepared in media, onto 96 well black bottomed plates (Nunc, Denmark) and allowed to attach for 24 hours. Six independent experiments were performed with six replicate wells for negative control, positive control and test concentrations on each plate. A working stock of 20 μ M DCFH-DA in PBS was prepared and all test concentrations, positive and unexposed negative controls were prepared in this working stock and exposed to cells. The negative control consisted of 20 μ M DCFH-DA in PBS only, the positive control was 5 μ M hydrogen peroxide (H₂O₂) prepared in DCFH-DA/PBS working stock and the AgNP test concentrations (3.91-500 μ g/ml) together with the AgNP-biofluid component test concentrations were prepared in the DCFH-DA/PBS working stock. Prior to cellular testing, acellular studies were performed with the test particles and the DCF dye at all test scenarios to confirm no reduction of the dye due to AgNP interference. No reduction of DCFH-DA by AgNP was noted (Gupta Mukherjee *et al*, 2012).

After 24 hours of attachment, the media was removed and wells were washed with PBS, 100 μ l per well. Cells were then treated with 100 μ l of positive control, negative control and test concentrations and plates were incubated for 15min, 30 min, 1hr, 2hr, 3hr, 4hr,

5hr and 6hr. The rate of intracellular ROS production was monitored at each time point by the emission of DCFH-DA at 529nm by excitation at 504nm at the various time points (plates were re-incubated after each time point reading). Readings were performed on a TECAN GENios plate reader (Grodig, Austria).

3.5.4 Confocal Microscopy

The confocal microscope enables visualisation deep within living tissues and cells. It uses point illumination and a spatial pinhole to eliminate out of focus light allowing only light within the focal plane to be detected. This allows for increased contrast and the ability to produce 3-dimensional structures from the images obtained.

Confluent cells were trypsinized and at a concentration of 1×10^5 cells per ml, 100 μ l of cell suspension were seeded onto 35mm glass bottom dishes (MatTek Corporation, Ashland, MA, USA). Glass dishes were incubated for 1 hour. Following incubation 1ml of cell culture media was added to cell suspensions. Dishes were re-incubated and allowed to attach for 24 hours. A working stock of 10 μ M CM-H₂DCFDA (Molecular Probes- Invitrogen, California, USA) was prepared in DMSO. A stock suspension of AgNP was prepared aseptically, from which different concentrations of nanoparticles could be prepared, was prepared in cell media followed by bath-sonication (Degussa-Ney ULTRASONIK 57X, California, USA). As a positive control, 10% DMSO was prepared in the respective cell culture media. The negative control consisted of cell culture media only.

After 24 hours of attachment, media was removed and glass dishes were washed with PBS 1ml per dish. Cells were treated with 100 μ l of CM-H₂DCFDA and incubated for 30 minutes. After incubation, dishes were washed in PBS 1ml per dish. 1ml test concentrations, negative and positive controls were added to dishes and incubated for 1 hour. After incubation dishes were washed in PBS 1ml per dish and imaged immediately. Images were taken on a Zeiss 510 LSM confocal microscope with an external argon ion laser source of 488nm excitation and using a bandpass filter of 505-530nm to detect the fluorescent DCF dye.

3.5.5 Clonogenic assay

The clonogenic assay, standardised by Franken *et al* (2006), was initially developed in 1956 by Puck and Marcus. The assay was performed in six well plates (Nunc, Denmark) each seeded with 1ml of cell suspension in media, 400 cells per ml, and 1ml of media alone. Cells were allowed to attach for 12 hours to ensure there were single cells at the bottom of each well at the time of exposure. After the attachment period, wells were washed with PBS 2ml per well and then treated with increasing concentrations of AgNP (3.91-500µg/ml) prepared in RPMI or AgNP prepared in RPMI with DPPC, 2ml per well. Plates were then incubated for 10 days, 37°C 5% CO₂ in a humidified incubator (Herzog *et al*, 2007; Casey *et al*, 2008). Two replicate wells on each plate were used for controls. This was performed for each test concentration. Following incubation, test exposures and controls were removed and wells washed with 2ml per well PBS. Finally cells were fixed and stained with 20% carbol fuchsin in dH₂O (BDH, Poole, UK) and colonies were manually counted. For all assays 10% DMSO prepared in supplemented media was used for positive control.

3.6 Inflammatory Studies

3.6.1 IL-8 & TNF- α

Release of inflammatory markers Interleukin 8 (IL-8) and tumour necrosis factor alpha (TNF- α) were studied using Human ELISA Max™ deluxe sets for IL-8 and TNF- α of BioLegend Inc. (San Diego, California). Supernatant samples were collected prior to inflammatory studies. Sample collection was performed in six well plates (Nunc, Denmark) each seeded with 1ml of cell suspension in RPMI, 5×10^4 cells per ml. Plates were incubated at 37°C for 24 hours. After incubation cells were treated with 1ml of increasing concentrations of AgNP (3.91-500µg/ml). Plates were incubated and cell supernatant collected at designated time intervals, 1 through 6 hours. After sample collection inflammatory studies were performed as per manufactures instructions. All reagents and standards were prepared as per instructions and all reagents were brought to room temperature prior to use. In brief, one day prior to running ELISA 100µl of capture antibody (IL-8/TNF- α) was added to each well of a Nunc Maxisorp™ 96 microwell plate. Plates were sealed and incubated for 16-18 hours at 4°C. Following capture antibody attachment, plates were washed 4 times with at least 300µl wash

buffer per well with residual buffer removed by firmly tapping plate on absorbent paper. To block non-specific binding 200µl assay diluent A was added to each well. Plates were sealed and incubated at room temperature for 1 hour with shaking at 200rpm. Following incubation, plates were washed as per instructions and 50µl of samples and standards were added to wells. Plates were sealed and incubated at room temperature for 2 hours with shaking. Following incubation plates were washed in wash buffer and 100µl detection antibody solution was added to each well, plates were then sealed and incubated at room temperature for 1 hour with shaking. Plates were washed in wash buffer and 100µl of Avidin-HRP solution was added to each well. Plates were sealed and incubated for 30 minutes at room temperature with shaking. Following incubation, plates were washed in wash buffer and 100µl TMB substrate solution C was added to each well with incubation in the dark for 15 minutes. After incubation, 100µl stop solution was added to each well and absorbance was read at 450nm and 570nm within 30 minutes using a SpectraMax® M3 Microplate reader (Molecular Devices, California, USA).

3.7 Pro-inflammatory Cytokine Gene Expression Analysis

3.7.1 Cell Line Treatment with AgNP

The human monocyte cell line THP-1 was cultured in RPMI 1640 (Sigma-Aldrich, Ireland) supplemented with 12.5% foetal bovine serum and 2mM L-glutamine. Cells were incubated at 37°C in humidified 5% CO₂. For exposures, cells at density of 1×10^5 cells per ml in 10mls cell culture media were seeded in T₂₅ flasks (Corning Life Sciences) and incubated overnight at 37°C in humidified 5% CO₂. Designated flasks were then exposed to either two concentrations of AgNP (50 and 100µg/ml), LPS (1µg/ml) or a combination of AgNP and LPS. AgNP and LPS and a combination of AgNP and LPS in cell culture media were added to assigned flasks making a final volume of 20mls per flask. Flasks were incubated for 1, 6, 8 or 24 hours. Following exposures cell suspensions were centrifuged at 1200rpm for 10 minutes at 4°C. Supernatants were removed and stored for further analysis and cells were re-suspended in 1ml PBS ready for RNA extraction.

AgNP exposure doses for later gene expression studies were chosen based on Inhibitory Concentration (IC₅₀) of THP-1 cells exposed to increasing concentrations of AgNP (1.9-

250µg/ml) as determined by the alamar blue (AB) cytotoxicity assay. Cells were seeded in 96 well microtitre plates (Nunc, Denmark) at a density of 1×10^5 cells/ml for 24 hour exposure in 50µl cell culture media, after which designated flasks were exposed to 50µl of increasing concentrations of AgNP. At least three independent experiments were conducted with six replicate wells employed per concentration per plate in each independent experiment. Plates were treated with increasing concentrations of AgNP prepared in media for 24 hours. This time point was chosen as gene studies require shorter exposure times in order to detect gene expression. Following incubation the assay was performed according to manufacturer's instructions. Briefly, 50µl AB solution was added to wells (total volume 150µl) and incubated for 3 hour. Following incubation AB fluorescence was measured at excitation and emission wavelength of 531nm and 595nm respectively using a SpectraMax® M3 Microplate reader (Molecular Devices, California, USA). Wells containing AB solution and media only were used as blanks with 10% DMSO used as a positive control. For this assay the mean fluorescence units for six replicate cultures were calculated for each exposure treatment. IC₅₀ values were calculated from the average response of three independent experiments fitted to a sigmoidal curve and a four parameter logistic model used to calculate IC₅₀ with a ($p < 0.05$).

In an experiment to establish possible endotoxin contamination of AgNP, THP-1 cells were incubated with polymyxin B (20µg/ml) for 1 hour prior to AgNP exposure. Cells at density of 1×10^5 cells per ml in 10mls cell culture media were seeded in two sets of four (eight total) T₂₅ flasks (Corning Life Sciences) and incubated overnight at 37°C in humidified 5% CO₂. One set of flasks were incubated with 20µg/ml polymyxin B for 1 hour prior to exposure. Designated flasks were then exposed to either AgNP (50 µg/ml), LPS (1µg/ml) or a combination of AgNP and LPS. AgNP and LPS and a combination of AgNP and LPS in cell culture media were added to assigned flasks making a final volume of 20mls per flask. Flasks were incubated for 8 hours. Following exposures, cell suspensions were centrifuged at 1200rpm for 10 minutes at 4°C. Supernatants were removed and cells were re-suspended in 1ml PBS ready for RNA extraction and subsequent gene expression analysis. The presence of polymyxin B prevented LPS induced gene expression but did not inhibit AgNP induced gene expression. Endotoxin was not detected in AgNP used in this study.

For IL-1 β ELISA studies, THP-1 cells (n=5) were plated alone or stimulated with LPS (10ng/ml) for 4 hours and exposed to ATP (5mM) for 1 hour or AgNP (50 and 100 μ g/ml) for 6 hours at 37°C. In some cases cells were pre-treated with Ac-YVAD-CMK (20 μ M) for 1 hour prior to ATP and AgNP exposures.

3.7.2 Primary Monocyte Isolation and Treatment with AgNP

20mls of whole blood were taken from 20 normal healthy donors after acquiring internal ethical approval via a review board and individual donor consent in heparinized vacutainers and all samples were processed immediately. Monocytes were extracted by positive selection process using CD14 Dynabeads® from Invitrogen by Life Technologies (Dublin, Ireland). Prior to use beads were washed and re-suspended as per manufacturer instructions. For the isolation process 500 μ l beads per 20mls whole blood were utilized and the procedure was performed as per instructions. Briefly, whole blood samples were diluted 1:2 in monocyte isolation buffer and centrifuged at 600g for 10 minutes at room temperature. Following centrifugation the upper layer was discarded and the blood re-suspended to the original volume in monocyte isolation buffer. Blood was transferred to 15ml tubes and 500 μ l Dynabeads® were added to each sample. Tubes were incubated for 20 minutes at 2-8°C with gentle tilting. Following incubation, tubes were placed in the DynaMag™ 15 magnet (Invitrogen by Life Technologies, Dublin, Ireland) for 2 minutes. For positive monocyte selection, the supernatant was carefully removed and discarded from the tubes which remained in the magnet and had attached monocytes in the tube. 15ml isolation buffer was added to tubes with gentle mixing to wash monocytes from the tubes. This wash step was repeated twice more to obtain a high purity of extracted monocyte cells. The pellet was then re-suspended in RPMI-1640 cell culture medium.

Cells were counted on a Beckman Z™ Series COULTER COUNTER® (Brea, California, USA) and were seeded at a concentration of 4×10^4 cells per ml (500 μ l total volume) in a 24 well plate (Nunc, Roskilde, Denmark), and incubated at 37°C with 5% CO₂ overnight. Cells were then exposed to AgNP concentration of 50 μ g/ml (IC₅₀ concentration). Plates were incubated for 8 hours at 37°C with 5% CO₂. Experimentation was limited on the extracted monocytes as only as small number of monocytes was harvested per donor blood sample. Therefore all optimisation

experiments incorporating concentration, time and the effects of LPS stimulation were carried out on THP-1 cells prior to these experiments.

Following incubation cells were removed from the base of the 24 well plates using a cell scraper (Sarstedt, Numbrecht, Germany), suspended in cell culture media, and spun in a pre-cooled microfuge at 3000g for 5 minutes at 4°C. Cell pellets were re-suspended in 1ml PBS for subsequent RNA extraction.

3.7.3 Gene Expression Analysis

The ability of AgNP exposure to induce pro-inflammatory cytokine gene expression was evaluated using RT-PCR. THP-1 cells and monocytes from a human cohort (n=20) were cultured in vitro and exposed to AgNP as described in the previous section. Once cells were centrifuged and the pellet re-suspended, total RNA was isolated using the Tri-reagent protocol developed by Chomczynski & Sacchi (1987). Briefly, the pellet was re-suspended in a pre-cooled microfuge at 3000g for 5 minutes at 4°C. The supernatant was removed and 1ml of Tri-reagent added. Samples were allowed to stand at room temperature for 5 minutes then 0.2ml of 1-Bromo-3chloropropane per ml of Tri-reagent was added. Samples were mixed for 15 seconds and allowed to stand for 2 minutes at room temperature before centrifugation at 12000g for 15 minutes at 4°C. The aqueous phase (colourless upper liquid) was transferred to a fresh tube. 0.5ml of isopropanol per ml of Tri-reagent was added to each tube, and incubated at room temperature for 5 minutes. Tubes were centrifuged at 12000g for 10 minutes at 4°C, the supernatant was removed and the pellet was washed with 1ml ethanol. The RNA pellet was re-suspended in 30µl of DEPC H₂O. First strand cDNA was synthesized using qScript™ kit Quanta Bioscience (Maryland, USA), according to manufactures instructions. Briefly, reverse-transcription master mix was prepared; 15µl of master mix was pipetted into individual tubes followed by 5µl each RNA sample. Tubes were mixed gently and contents were collected by brief centrifugation. Tubes were placed in a Thermocycler following the protocol and the run was initiated.

The resulting cDNA was amplified using quantitative real time PCR using LightCycler® 480 SYBR Green I Master from Roche Life Science (Sussex, UK). Samples were analysed on LightCycler® 480 white 96 well plates from Roche Life Science (Sussex, UK).

Table 1 Table of forward and reverse primer sequences for housekeeping gene actin, and for target genes IL-1, IL-6 and TNF- α

Gene	Forward Sequence	Reverse Sequence
Actin	5'-ACTCTTCCAGCCTTCCTTCC	5'-GTTGGCGTACAGGTCTTTGC
IL-1	5'-GGGCCACACATCTACTAGGC	5'-TGGGTATCTCAGGCATCTCC
IL-6	5'- GATGCAATAACCACCCCTGACCC	5'- CAATCTGAGGTGCCCATGCTAC
TNF- α	5'-AAGAGAATTGGGGGCTTAGG	5'-CAGGGATCAAAGCTGTAGGC

Cycling conditions were the same for all primers and as follows: 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds, followed by 1 cycle of melting curve of 95°C for 5 seconds, 65°C for 1 minute then cooling at 4°C. PCR was performed on a Roche LightCycler®480 system (Sussex, UK). CT data results were obtained directly from the LC480 program (using SYBR green fluorescence) and used to determine the pro-inflammatory gene expression levels following AgNP exposure. The delta delta ($\Delta\Delta$) CT method of relative quantification was applied in the analysis of RT-PCR gene expression data (Livak & Schmittegen, 2001).

3.7.4 Human IL-1 β ELISA

Release of Human IL-1 β was studied using a Human IL-1 β ELISA Max™ Deluxe set from BioLegend Inc. (San Diego, CA, USA). THP-1 cells were plated in 24 well plates (Nunc, Roskilde, Denmark) 2×10^5 cells per well in 200 μ l of 10% FBS 1640 RPMI medium. AgNP in cell culture media were added to each well making a final volume of 400 μ l per well. Cell culture supernatant was collected after 6 hours and stored at -80°C. ELISA carried out as per manufacturer instructions. Briefly, one day prior to investigation 96 well plates were coated with diluted Capture Antibody solution, 100 μ l per well. Plates were sealed and incubated overnight between 2 and 8°C. The following day plates were washed 4 times with wash solution as per instructions and 200 μ l 1X Assay Diluent A was added to each well in order to block plates. Plates were sealed and

incubated at room temperature for 1 hour with shaking. After incubation plates were washed 4 times and 50µl of Assay Buffer D was added to wells that would contain sample or standard. 50µl of sample or standard was added to the appropriate wells and plates were sealed and incubated at room temperature for 2 hours with shaking. Following incubation, plates were washed 4 times and 100µl of diluted Detection Antibody solution was added to each well. Plates were sealed and incubated at room temperature for 1 hour with shaking. After incubation plates were washed 4 times and 100µl of diluted Avidin-HRP solution was added to each well. Plates were sealed and incubated at room temperature for 30 minutes with shaking. Following incubation plates were washed 5 times with soaking for 30 to 60 seconds per wash. 100µl of Substrate Solution was added to each well and plates were incubated in the dark for 20 minutes. After incubation 100µl of Stop Solution was added to each well to stop the reaction. Absorbance was measured immediately at 450nm and 570nm using a SpectraMax® M3 Microplate reader (Molecular Devices, California, USA).

3.8 Statistical analysis

At least three independent experiments were performed for each cytotoxicity endpoint. Results for each assay were expressed as a percentage of unexposed control \pm standard deviation with control values set as 100%. Statistically significant differences between samples and their respective controls were calculated using the statistical analysis package InStat (GraphPad Software Inc, San Diego, California). Statistically significant differences were set at $p < 0.05$. Normality of data was confirmed with Q-Q percentile plots and Kolmogorov-Smirnov tests. Equality of variances was evaluated using Levène tests. One-way analysis of variances (ANOVA) followed by Dunnett's multiple comparison tests were carried out for normally distributed samples with homogeneous variances. Non-parametric tests, namely Kruskal-Wallis followed by Mann-Whitney-u-tests were applied to samples without normal distribution and/or inhomogeneous variances. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a four parameter logistic model used to calculate the Inhibitory Concentration (IC_{50}) values. The IC_{50} value refers to a concentration of a compound where a 50% effect is observed, in this case a reduction in cell viability. IC_{50} values were reported as $\pm 95\%$ confidence intervals. IC_{50} values were estimated using XLfit3™, a curve fitting add on for Microsoft® Excel (ID Business Solutions, UK).

For real-time PCR experiments, relative quantification analysis directly from the LC480 program (from the real-time PCR experiments using SYBR green fluorescence) was used to determine the pro-inflammatory gene expression levels following AgNP exposure. The reference gene (Actin) normalises sample to sample differences and was important to determine the changes in expression of different genes, according to concentration and time.

The quantitative endpoint for real-time PCR is the threshold cycle (CT). It is defined as “The PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold” (Schmittegen & Livak, 2008). A mathematical model widely used in the analysis of RT-PCR data is the delta delta ($\Delta\Delta$) CT method (Livak & Schmittegen, 2001). This was applied to the raw CT data to give a ratio of target gene to the control gene to demonstrate gene expression changes. Mean-fold changes are calculated from mean normalised values of raw CT data between samples. The $\Delta\Delta$ CT mathematical model normalises sample to sample differences. The aim was to determine the effect of AgNP on the pro-inflammatory cytokine gene expression. This method enabled the measurement of gene expression changes of target genes normalised to Actin (housekeeper gene) monitored at AgNP exposure (50 and 100 μ g/ml) at 1, 6, 8 and 24 hours relative to the expression without AgNP exposure (control). The value of the mean-fold change of gene expression without AgNP (control) was 1. The mean-fold changes in gene expression of each target gene were plotted by Microsoft Excel, and the graphs present either up-regulation or down-regulation of samples determined by whether they reach above or below the control sample value of 1.

Statistically significant differences between samples and their respective controls were calculated using the statistical analysis package InStat. Statistically significant differences were set at $p < 0.05$. Normality of data was confirmed with Q-Q percentile plots and Kolmogorov-Smirnov tests. Equality of variances was evaluated using Levene tests. One-way analysis of variances (ANOVA) followed by Dunnett’s multiple comparison tests were carried out for normally distributed samples with homogeneous variances. Non-parametric tests, namely Kruskal-Wallis followed by Mann-Whitney-u-tests were applied to samples without normal distribution and/or inhomogeneous variances.

3.9 Chapter Summary

This chapter has highlighted the various techniques employed to characterise the physical and chemical properties of AgNP and also the various assays used to determine the toxic effect (if any) of these particles on the chosen cell lines. The following chapters will discuss the results obtained using the techniques described here.

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Chapter 4

Physicochemical Characterisation of Silver Nanoparticles

Adapted from “Potential of biofluid components to modify silver nanoparticle toxicity” and “The surfactant dipalmitoylphosphatidylcholine modifies acute responses in alveolar carcinoma cells in response to low concentrationsilver nanoparticle exposure”

4.1 Introduction

Nano-metals, in particular silver nanoparticles (AgNP) have shown remarkable antimicrobial properties and as a result have been applied to various consumer products (Sozer & Kokini, 2009; Sotiriou & Pratsinis, 2011; Chen & Schluesener, 2008). The widespread contact with AgNP has raised concerns over their possible toxicity upon entering the body. Paramount to their proposed toxicity is their physicochemical characteristics including, size, shape and also their interaction with cell culture media and its various protein components. This chapter will explore the interaction of AgNP with biofluid components, ursodeoxycholic acid (UDCA), cholic acid (CA), and deoxycholic acid (DCA), three components of bile and dipalmitoylphosphatidylcholine (DPPC) a major component of human lung surfactant. These biofluid components were selected to correspond to the exposure route investigated in this report. Bile plays a central role in lipid digestion and absorption of nutrients while lung surfactant is essential for the normal breathing process (Maldonado-Valderrama *et al*, 2011; Seranno & Peres-Gil, 2006). The various biofluids play important roles within the body and it is essential that we establish how AgNP will interact with them, as inevitably they will come into contact with one another upon exposure. Before toxicity testing was performed the AgNP were fully characterised using Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) particle size analysis and zeta potential analysis. Where possible, measurements were performed on AgNP as purchased and on test suspensions of the same. The ability of the aforementioned biofluid components to alter particle size, aggregation and particle stability was monitored and discussed. The importance of establishing physicochemical characteristics of nanoparticles has been highlighted as a critical component when performing toxicity testing. As nanoparticle

characteristics will most likely change in cell culture media and biological systems from the pristine powder, performing a full physicochemical profile will provide insight into the toxic mechanisms of nanoparticles. Failure to adequately characterise nanoparticles will make any toxicological data insignificant, of limited value and will not be comparable to other studies with a similar nanomaterial (Warheit, 2008). In light of this observation a full physicochemical profile of AgNP will follow, first focusing on particle size and any alterations from the pristine powder when in suspensions of cell culture media and in the presence of biofluid components.

4.2 Particle Size

The methods of particle characterisation employed to determine the relative size of AgNP as purchased and in various suspensions were SEM and DLS. Figure 4.1 (a) and (b) shows SEM analysis of a dilute sample of AgNP. This sizing method was performed on pristine particles as purchased from Sigma-Aldrich (Catalogue No: 758329). SEM revealed spherical particles with a size of 50-70nm. This method reflects the physical particle diameter of the pristine particles as purchased. In a study published by Gupta Mukherjee *et al* (2012), SEM analysis determined a diameter of <50nm for the same Sigma-Aldrich AgNP employing the same preparation method for SEM analysis as this study. While this method of particle sizing provides useful information on the pristine AgNP, it does not represent how particles will be presented to biological systems upon entering the body and how the various biofluids they come into contact with may affect them. While SEM analysis provides a fast analysis of nanoparticles with a simple sample preparation, analysis of biological samples by this method requires fixation and any change in nanoparticle size due to interaction with biological material cannot be monitored by this method. DLS analysis will allow monitoring of the system AgNP are suspended in and provide data on particle hydrodynamic radius and any change that occurs due to the presence of certain biological material, in this case protein components of cell culture media, biofluid components and the surfactant DPPC.

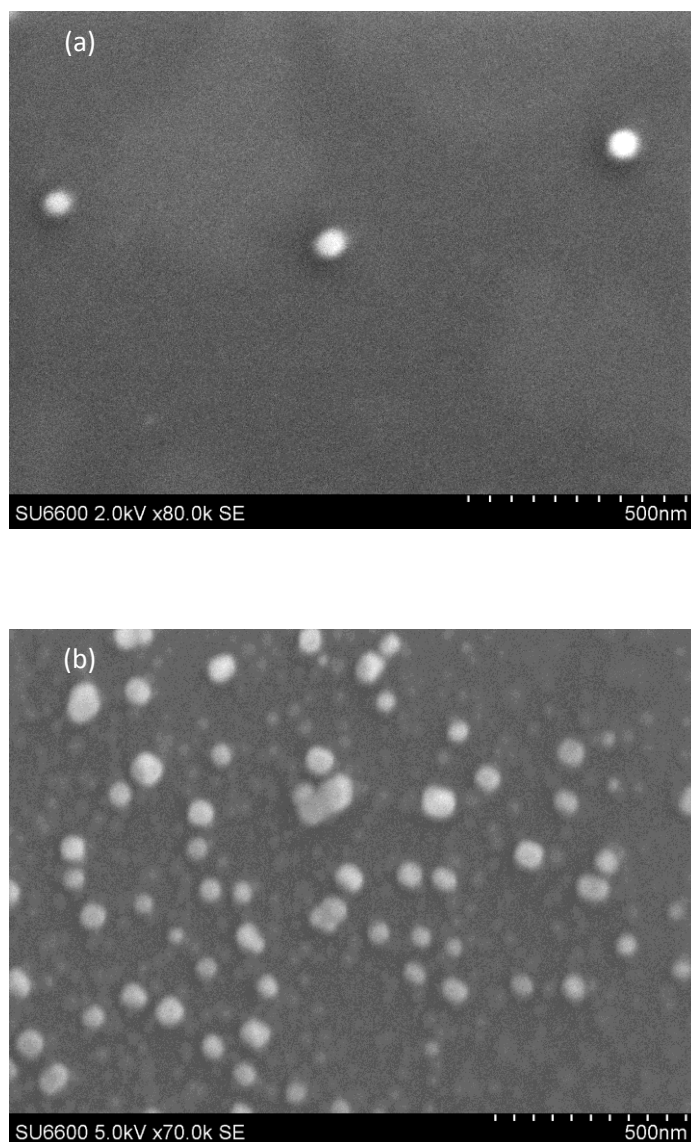


Figure 4.1 (a) and (b) SEM micrograph at scale bar of 500nm image pristine AgNP (a) at a magnification of x80,000 and (b) at a magnification of x70,000 dispersed in ethanol to a concentration of 1.56 μ g/ml by sonication using Ultrasonic Processor tip

4.3 DLS Analysis of AgNP with Biofluid Components UDCA, CA and DCA

DLS size measurements were performed on pristine AgNP suspended in dH₂O and cell culture media with and without the addition of the tested biofluid components to the media. A concentration of AgNP was chosen for DLS analysis of 15.6 μ g/ml representing a concentration at which a toxic effect was observed, as will be discussed in chapters 5 and 6. DLS data from this study is illustrated in figure 4.2 (a)-(d). When suspended in a biological media the various proteins within that media can associate

with any nanoparticles present and it is postulated that this protein coating can determine the response of biological systems rather than the particles themselves (Lynch *et al*, 2007). It can be assumed that any protein association that occurs in the nanoparticle dispersions presented here may impact the hydrodynamic radius of particles and cause possible agglomeration.

From DLS analysis, AgNP size in dH₂O was determined to be 34nm \pm SD 3.5nm. A shift in hydrodynamic radius was observed in preparations of AgNP in RPMI. Dispersed in RPMI alone there are two clear peaks observed one representing RPMI itself and the other at approximately 40nm representing AgNP. Upon addition of UDCA to the dispersion no effect on the hydrodynamic radius was noted. The two peaks were still observed with only a slight increase in size distribution. This variation may be explained by a shift in the plane of shear upon addition of UDCA as UV-Vis spectroscopic analysis demonstrated no significant interaction of AgNP with the cell culture media components. In the presence of DMEM there was a shift in the distribution of AgNP compared to their dispersion in dH₂O. The first peak remained relatively unchanged apart from a reduced number of particles falling within this size range. The second wider peak demonstrated the greatest change with the majority of particles falling within this size range which has shifted to below 100nm. However upon addition of UDCA, CA and DCA (figure 4.2 (b) – (d)) significant changes were noted with only one peak between 1-10nm observed which was attributed to DMEM. UDCA, CA and DCA have a large size distribution (greater than 200nm) and may result in the formation of large agglomerates with nanoparticles, causing them to precipitate out at an elevated rate preventing their detection by DLS analysis resulting in the detection of DMEM only. A possible reason may be due to masking of particles in larger agglomerates caused by the presence of CA and DCA or potentially the particles may have fallen out of solution at a faster rate following addition of these biofluid components preventing their detection. Further investigation is required into why these particular dispersions of AgNP and biofluid components cannot be detected by DLS analysis and how this will ultimately affect their toxicological testing. This may result in altered toxic responses due to association of biofluid components with particles. This will be discussed in more detail in chapters 5 and 6.

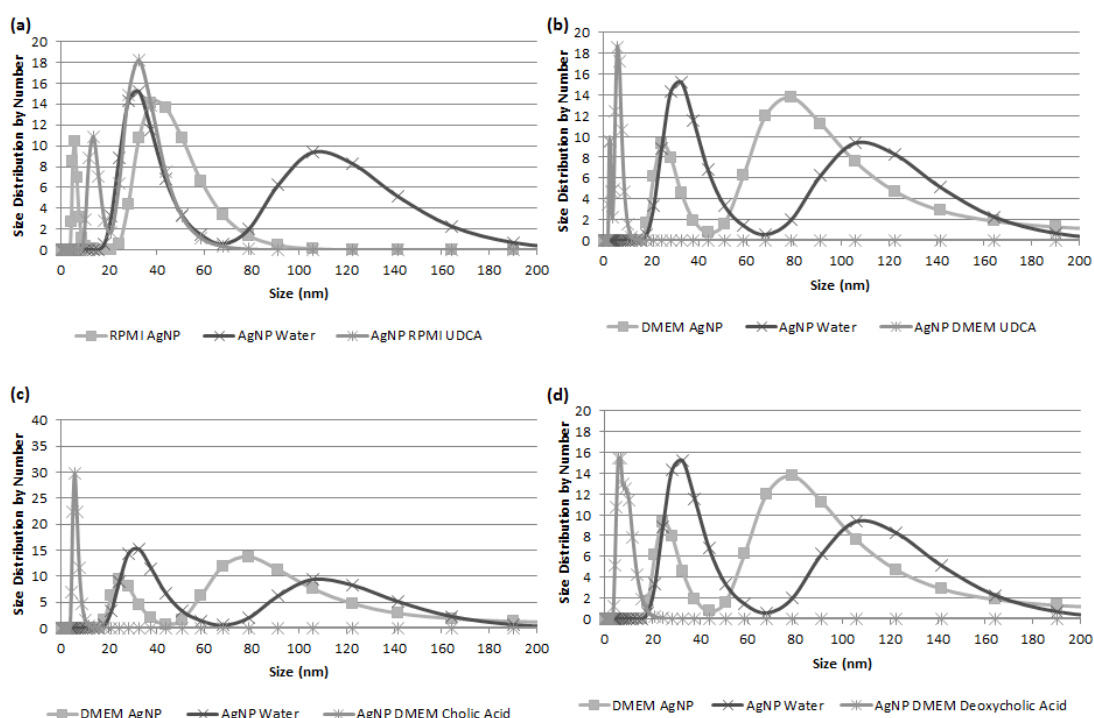


Figure 4.2 (a) – (d) Dynamic Light Scattering size (nm) particle number (count) distribution plot of AgNP (15.6µg/ml) dispersed in media, dH₂O and in the presence of (a) UDCA (RPMI), (b) UDCA (DMEM), (c) CA and (d) DCA. Data presented is the average of three individual experiments

4.4 DLS Analysis of AgNP with DPPC

Figure 4.3 demonstrates DLS measurements on AgNP suspended in dH₂O, in RPMI cell culture media and in the presence of DPPC. A representative concentration of AgNP (15.6µg/ml) that produces a biological effect was chosen. Two peaks were demonstrated in analysis of AgNP alone with the greatest number of particles falling in the range of 34nm ±3.5. The broader peak can be attributed to particle agglomeration. A slight shift in hydrodynamic radius was observed in preparations of AgNP in RPMI. Dispersed in RPMI alone two clear peaks were observed one representing RPMI itself and the other at approximately 40nm representing AgNP. The addition of DPPC caused little change in hydrodynamic radius but produced a larger peak at the 20-40nm range suggesting possible isolation of particles from larger agglomerates with its addition to the system.

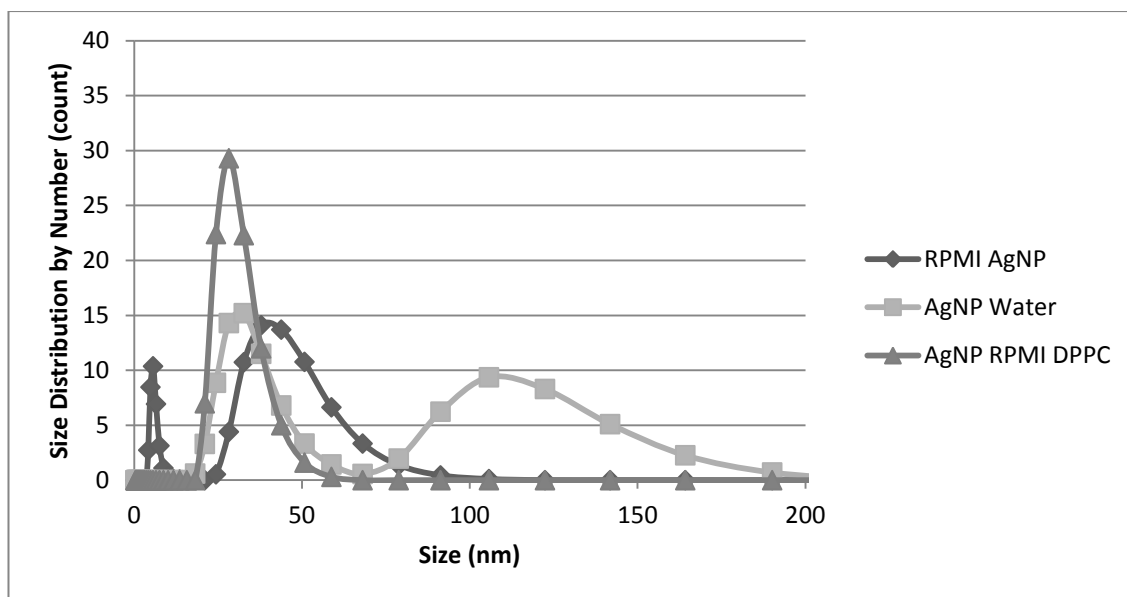


Figure 4.3 Dynamic Light Scattering size (nm) particle number distribution plot of AgNP (15.6 μ g/ml) dispersed in media, dH₂O and in the presence of DPPC. Data presented is the average of three individual experiments

4.5 UV-Vis Spectroscopy

UV-Vis absorption spectroscopy was performed to identify the features of the cell culture media RPMI and DMEM and of AgNP dispersed in the culture media. This method was employed to explore if AgNP interact with cell growth medium and its various constituents. The UV-Vis spectrum of cell culture media consists of a number of features at 360, 410 and 560nm. There is also a feature at 270nm that is not illustrated. The feature at 410nm is the 10% foetal bovine serum (FBS) that is added to culture media which also displays another feature at 270nm, the feature at 360nm is riboflavin, a vitamin present in the medium and finally the feature at 560nm can be attributed to the phenol red indicator.

The UV-Vis absorption analysis of the different preparations of AgNP in cell culture media and with the addition of biofluid components demonstrated no changes to the spectra. The lack of alteration suggests that secondary toxicity caused by nutrient depletion would be unlikely. Data are presented in Appendix A4.4.1 (a) and (b).

While DPPC appeared to have no influence on particle size the data indicate an influence on particle agglomeration. As such UV-vis absorption was performed to

determine any interaction between the various components of the different suspensions. No interaction between components was demonstrated indicating that a secondary toxicity due to depletion of nutrients would be unlikely. Data are presented in Appendix A4.4.2.

4.6 Stability and Surface area

Zeta potential analysis, illustrated in Tables 4.1 and 4.2, was performed using the same sample preparations as for sizing analysis. Typically values above 30mV or below -30mV indicate stability of the overall system. Zeta potential analysis of AgNP dispersed in dH₂O (Table 4.1) showed instability of particles at all test concentrations which is unexpected as the particles purchased are PVP coated. When the particles were dispersed in the test media differences were noted and the AgNP in RPMI demonstrated stable results, whereas interestingly dispersion of AgNP in DMEM was revealed to be unstable. Upon the addition of the bile components CA, DCA and UDCA however, the system was also found to be unstable. The large values noted when particles were dispersed in RPMI and RPMI with UDCA would suggest a tendency to repel each other and as such reduce the likelihood of agglomeration. The lower values for AgNP in DMEM and DMEM with UDCA, CA and DCA suggest reduced repulsive forces between particles increasing the potential to agglomerate (Xia *et al*, 2006). This may result in particle precipitation out of solution as observed in DLS analysis. However it can be assumed from the data that the addition of biofluid components does not modify the overall stability of the system compared to the values noted for dispersions of AgNP in cell culture media alone.

Table 4.1 Table of Zeta Potential results for three concentrations of AgNP 3.91, 15.6 and 62.5 μ g/ml dispersed in different cell culture media and in the presence of different biofluid components. Data expressed as average value \pm SD of three individual experiments.

	62.5 μ g/ml	15.6 μ g/ml	3.9 μ g/ml
AgNP Only	-15.5 \pm 1.2mV	-31.2 \pm 0.9mV	-22.1 \pm 6.6mV
AgNP RPMI	-473.7 \pm 50.8mV	-380 \pm 121.1mV	-221 \pm 117.2mV
AgNP DMEM	-7.9 \pm 5.1mV	1.9 \pm 3.4mV	-11.2 \pm 7.2mV
AgNP 1mM CA-DMEM	-7.1 \pm 6.9mV	-2.6 \pm 3.4mV	-27.6 \pm 5mV
AgNP 0.125mM DCA-DMEM	-4.9 \pm 9.8mV	-12.1 \pm 5mV	-5.5 \pm 8.9mV
AgNP 50 μ M UDCA-DMEM	-6.6 \pm 2.3mV	-8.1 \pm 6.4mV	-6 \pm 5mV

Zeta potential analysis was also performed on AgNP suspensions with DPPC as illustrated in Table 4.2. When suspended in cell culture media and in the presence of DPPC the system was stable. The addition of DPPC was noted to have no impact on the overall stability.

Table 4.2 Table of Zeta Potential results for three concentrations of AgNP 3.91, 15.6 and 62.5 μ g/ml dispersed in RPMI and in the presence of DPPC. Data expressed as average value \pm SD of three individual experiments.

	62.5 μ g/ml	15.6 μ g/ml	3.9 μ g/ml
AgNP Only	-15.5 \pm 1.2mV	-31.2 \pm 0.9mV	-22.1 \pm 6.6mV
AgNP RPMI	-473.7 \pm 50.8mV	-380 \pm 121.1mV	-221 \pm 117.2mV
AgNP 0.25 μ g/ml DPPC-RPMI	-454 \pm 175.6mV	-485 \pm 106.5mV	-440.3 \pm 434.5mV

BET analysis was also employed to determine surface area of AgNP powder as purchased and yielded a surface area of $2.3 \pm 0.1 \text{ m}^2/\text{g}$.

4.7 Discussion

The physicochemical characteristics of AgNP play an important role in how they will interact with biological systems. The size, associated surface area, shape and chemical composition have all been shown to be of great importance to nanoparticle toxicity. Size influences surface area which leads to greater cell contact, while shape can also influence interactions for example, truncated triangular nanoparticles show increased toxicity compared to spherical nanoparticles and it is believed that cylindrical particles can cross the cell membrane easier than other shapes due to their ability to harness the clatherin machinery (Renwick *et al*, 2004; Rai *et al*, 2009; Kam *et al*, 2004).

It is now well established that upon entry into the body nanoparticles almost immediately become coated in protein and that this adsorbed layer or “corona” is important in biological responses to nanoparticle exposure (Gray, 2004; Lynch & Dawson, 2008). While there will be adsorption of the proteins of biofluid components, DPPC and cell culture media onto the nanoparticle surface, it can be assumed that these proteins will associate and dissociate from AgNP at different rates depending on a factors such as concentration and affinity (Lynch *et al*, 2007).

Proteins tend to agglomerate upon dispersion in an aqueous solution and may coat AgNP trapping them within agglomerates increasing the rate at which they fall out of solution. A number of studies have investigated this dispersion to determine how this affects overall toxicity. It is known that nanoparticles once suspended in cell culture media can precipitate out of solution and agglomerate at different rates. As such this raises a number of questions relating to dosimetry and the effect of agglomerate formation on the end result of nanoparticle exposure. As well as influencing the results of toxicity it can also influence exposure times as agglomerate formation and precipitation may increase the time taken for delivery of the full concentration to cells. Further investigation is required to develop a method of including dosimetric parameters into toxicological testing so that accurate results for *in vitro* assays can be obtained that are comparable to animal models (Cohen *et al*, 2014; Cohen *et al*, 2013; Kroll *et al*, 2009; Teeguarden *et al*, 2007).

Zeta potential analysis was also performed and a change in values would indicate interaction of nanoparticles with biofluid components, the surfactant DPPC and cell culture media, suggesting indirect toxicity caused by their interaction (Casey *et al*, 2007; Casey *et al*, 2008). No change on overall stability was noted following biofluid component or DPPC addition. The overall particle stability also influences size and agglomerative state. Larger unstable zeta potential values were noted for AgNP dispersions in RPMI with UDCA addition. These large values suggest particles will repel each other reducing the potential to agglomerate. The lower values noted for AgNP dispersions in DMEM with UDCA, CA, DCA suggest low repulsive forces and thus a tendency to agglomerate. This may explain the lack of particle size data due to particle precipitation out of solution potentially caused by agglomeration. The dispersant is an important factor in particle stability thus effecting size and possible agglomeration (Xia *et al*, 2006).

A recent study demonstrated that AgNP exposed to synthetic stomach fluid led to transformations in particles including particle aggregation, changes in particle morphology and the release of silver ions (Mwilu *et al*, 2013). It was suggested that alterations to AgNP size and surface properties due to exposure to synthetic stomach acid may impact their transformation through the gastrointestinal tract and may in turn influence AgNP interaction with the cells they encounter. The binding of proteins be it from cell culture media, biofluid components or other surfactants can aid in the binding of particles to cell surfaces, their trafficking through biological systems and the resulting effects. Very recently in a study by Wang *et al* (2013), the protein surface coating or “corona” was identified as providing a Trojan horse effect to nanoparticles. Initial entry of nanoparticles into the cell did not cause immediate toxicity it was only when the nanoparticles were transported to the lysosome and the surface coating was degraded in the acidic environment, that toxicity was observed. At this point, much later after initial exposure, loss of lysosomal integrity and release of its contents was noted followed by cell death via apoptosis (Wang *et al*, 2013). These findings can be related to the work undertaken in this study as biofluid components and the surfactant DPPC are protein entities and will ultimately form part of the protein corona and may contribute to particle interactions with living systems and any related toxicity which will be further discussed in chapters 5 and 6.

This reiterates the importance of performing realistic exposure scenarios in toxicological testing to determine nanoparticle interactions in biological systems. These results highlight the importance of establishing the physicochemical characteristics of AgNP in a variety of biological suspensions, as their interaction with these biofluids influence their interactions with cellular components and biological systems and will ultimately determine their distribution throughout the body and the signalling pathways and end responses they induce.

4.8 Chapter Summary

This chapter has illustrated the physicochemical characteristics of AgNP both as purchased and with the addition of biofluid components and the surfactant DPPC. The ability of the tested biofluid components and DPPC to influence AgNP size, aggregative state and stability were discussed. By gaining an understanding of these characteristics and how AgNP interact with biofluid systems within the body, may help in our understanding of how this contact may modify interaction with biological systems and any associated toxicity. The presence of biofluid components and surfactants may change how AgNP interact with biological systems, their distribution within the body, how a cell “sees” the particle and ultimately the response that results from particle-cell interaction.

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Chapter 5

Adapted from “Potential of biofluid components to modify silver nanoparticle toxicity”

5.1 Introduction

Nanotechnology has been described as a new frontier in science and technology with many different applications in fields including textiles, electronics, medicine, cosmetics and food (Sozar & Kokini, 2009; Bouwmeester *et al*, 2009). Silver nanoparticles (AgNP) in particular are becoming the material of choice for incorporation into consumer goods mainly due to their antimicrobial properties (Choi *et al*, 2008).

When considering nanoparticle exposure it is important to take into account not only the route of entry to the body but also the various biological systems, biofluids and surfactants they will encounter. Indeed as a result of the potential interaction with various components of biofluids, nanoparticles may be affected, ultimately changing how they interact with cells and the responses they evoke.

With the ever increasing use of nanoparticles in food products and with pending EU legislation enforcing that all forms of engineered nanoparticles present in food must be stated on food packaging ie. “nano-labelling” it is now more crucial than ever that the interaction between nanoparticles and living systems is thoroughly investigated (Nano and other Emerging Technologies Blog, 2013). In particular, the potential toxic effect of nanoparticles to the gastrointestinal (GI) tract and their interactions with various biofluids associated with this system must be determined.

The GI tract has been noted as a major area of AgNP deposition coupled with pathological responses. A number of studies have demonstrated damaged microvilli and intestinal glands, abnormal pigmentation and an increase in intestinal goblet cells following oral administration of AgNP in rats (Hadrup & Lam, 2014; Shahare & Yashpal, 2013; Jeong *et al*, 2010; Kim *et al*, 2010). The liver in particular is an important site of nanoparticle deposition following ingestion with reports of high concentrations of AgNP reported in mice 24 hours after exposure. Inhalation also provides a route to the liver. Mucocilliary clearance of nanoparticles can result in entry to the GI tract and deposition in the liver. Accumulation of AgNP can lead to liver

toxicity including bile duct hyperplasia and inflammation (Kim *et al*, 2008; Nemmar *et al*, 2002). The liver has also been identified as a one of the main pathways involved in nanoparticle excretion. Hepato-biliary secretion is believed to be a main route of intestinal secretion of nanoparticles with evidence of gold and polystyrene nanoparticle excretion by this route. Accumulation of nanoparticles within bile canaliculi is suggestive of the important role of bile in the intestinal secretion and elimination of nanoparticles in faeces (Zhao *et al*, 2014; Johnston *et al*, 2010; Semmler-Behnke *et al*, 2008).

A biofluid is any fluid originating from within the body that can be excreted, secreted, obtained by a needle or can develop due to a pathological process and ranges from blood to bile and breast milk to cyst fluid (Medicinenet). It has been shown that certain biofluids and surfactants can aid the processing of nanoparticles and can isolate nanoparticles from larger agglomerates by coating the particle or modifying its surface chemistry (Herzog *et al*, 2009). Interactions with various surfactants can result in adsorption of a variety of different molecules and proteins onto the surface of nanoparticles. As a result, many studies have shown that this “coating” can affect how a nanoparticle interacts with the biological environment. These affects range from changes in the particle itself such as ion release and changes in morphology to the responses it induces and where it is trafficked within the body (Herzog *et al*, 2009; Mwilu *et al*, 2013; Ehrenberg *et al*, 2009; Wang *et al*, 2013; Misra *et al*, 2012; Aggarwal *et al*, 2009). It is this ability of biofluids to interact and process foreign bodies that make them integral to the defence systems of the body. In this study three components of bile were chosen and are referred to as biofluid components throughout as they were studied individually. The biofluid components employed were cholic acid (CA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA).

Bile salts are organic solutes synthesized from cholesterol by hepatocytes (Kumar & Bohidar, 2010; Perez & Briz, 2009). Cholic acid is one of the major primary bile acids in the liver and is synthesized from cholesterol and constitutes about 30-40% of bile acids (Debruyne *et al*, 2001). Formation of these bile acids is important in cholesterol homeostasis. Deoxycholic acid is a secondary bile acid and constitutes roughly 10-40% of total bile (Marcus & Heaton, 1988). DCA has also been demonstrated to promote colonic epithelium proliferation (Deschner *et al*, 1981; Ochsenkuhn *et al*, 1999). UDCA is a secondary bile acid comprising 3% of total bile acids (Perez & Briz, 2009). Despite

its low abundance it has been shown to have direct antioxidant properties and can prevent the retention of certain toxic hydrophobic bile acids. UDCA has also a number of therapeutic applications including dissolving gall stones and has shown promise as a targeted cancer therapy (Arisawa *et al*, 2009; Lapenna *et al*, 2002; Debruyne *et al*, 2001).

This study aims to demonstrate if biofluid components can modify nanoparticle toxicity. Their incorporation may enhance or reduce cytological effects and has often been overlooked in cytotoxicity screening, which will be addressed here. The European Union define a nanomaterial as “*A natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or as an agglomerate, and where for 50% or more of the particles in the number size distribution, one or more external dimensions is in the range 1-100nm*” (European Commission, 2011). In keeping with this definition it is important to investigate the interaction of biofluid components with nanoparticles to determine if the chemistry is affected resulting in a change in size distribution or agglomerative state potentially causing the nanoparticles to fall outside the criteria of a nanomaterial as per the EU definition. This was investigated and previously discussed in chapter 4. The cell lines chosen were HepG-2 a hepatocellular carcinoma (liver) and Hep2 an epithelial cell line employed as a control. A cytotoxic profile of AgNP was carried out using the standard cytotoxic assays 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and Alamar Blue (AB) viability assays. A 2', 7'-dichlorofluorescein diacetate (DCFH-DA) plate assay was used to detect any intracellular ROS generation in response to AgNP exposure.

5.2 Cytotoxicity Testing

Viability assays were performed to determine the effect of AgNP exposure to HepG-2 and Hep2 cell lines. The ability of the biofluid components UDCA, CA and DCA to modify the toxic response of AgNP was also evaluated. Cytotoxicity data for AgNP exposure in the HepG-2 and Hep2 cell lines are presented in table 5.1. The results of the MTT assay are presented for each cell line in the presence and absence of the respective biofluid component. Both MTT and AB assays were performed for each cell line. Results for AB assay are presented in Appendix A5.2.

5.2.1 Cytotoxic evaluation HepG-2 cell line to AgNP exposure alone and in the presence of UDCA, CA and DCA

Figure 5.1 represents the cytotoxic response of HepG-2 cells to AgNP exposure determined by the MTT assay. In the presence of AgNP cell viability decreases in a concentration and time dependent manner, with a substantial decline in mitochondrial integrity detected at concentrations of 15.6 µg/ml and above. A significant decrease in cell survival occurs at concentrations of 15.6 µg/ml after 48 hour exposure while a significant reduction in cell viability at all exposure time points occurs at AgNP concentrations of 31.25 µg/ml and upwards.

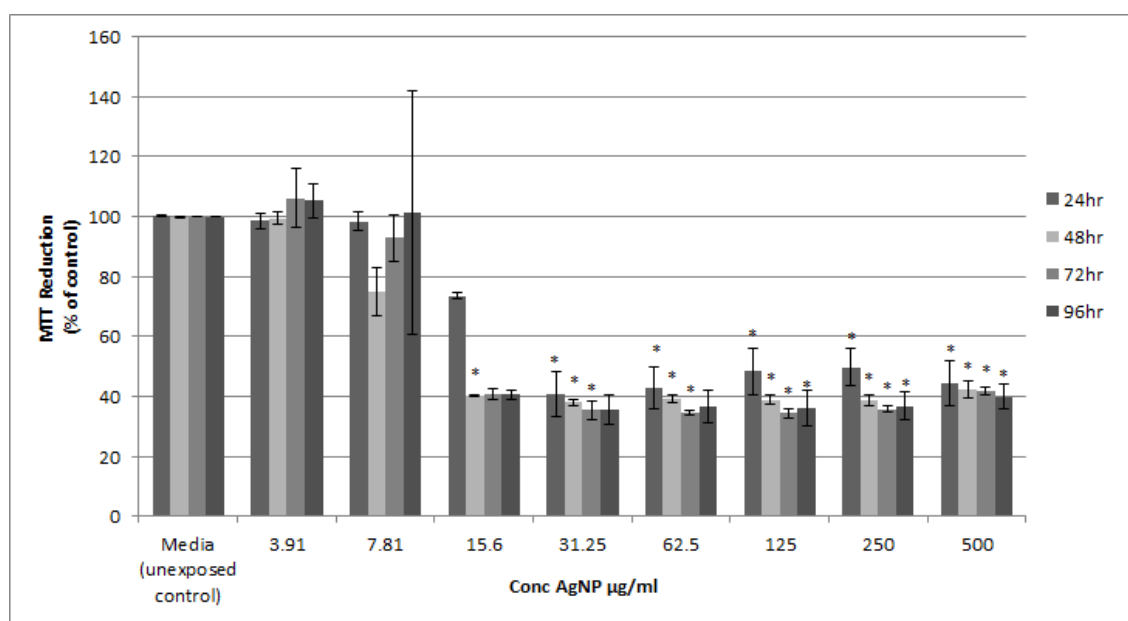


Figure 5.1 Cytotoxicity of AgNP in HepG-2 cells after 24, 48, 72 and 96hr exposures as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

A DCF-DA plate assay was employed to detect any ROS production in response to AgNP exposure over a 24 hour period. Readings were taken every hour for 6 hours. Following this, readings were then taken at 12 and 24 hours to detect any delayed intracellular ROS production in response to increasing concentrations of AgNP. Figure 5.2 illustrates ROS generation in response to AgNP exposure. The study showed that

the amount of ROS produced increased with increasing length of nanoparticle exposure and also with increasing concentration. ROS production was noted at concentrations of 7.8 μ g/ml and above after 15 minutes exposure and was shown to increase with concentration and exposure time. The greatest level of ROS production was observed at a concentration of 500 μ g/ml after 12 hours of exposure. A reading at 24 hours showed a decline in ROS production compared to the 12 hour time point which can be associated with cell death. Confocal microscopy was employed to confirm ROS induction due to AgNP exposure at a concentration of 31.25 μ g/ml in figure 5.3.

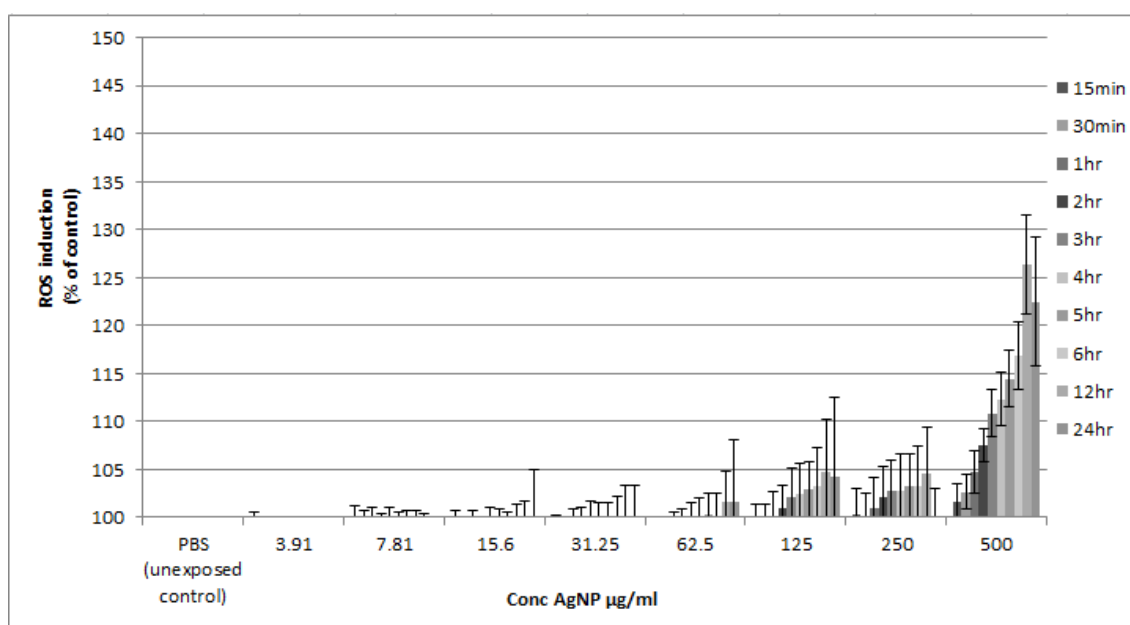


Figure 5.2 ROS generation in HepG-2 cells after different time points of AgNP exposure. Data expressed as percentage of control mean \pm SD of six independent experiments.

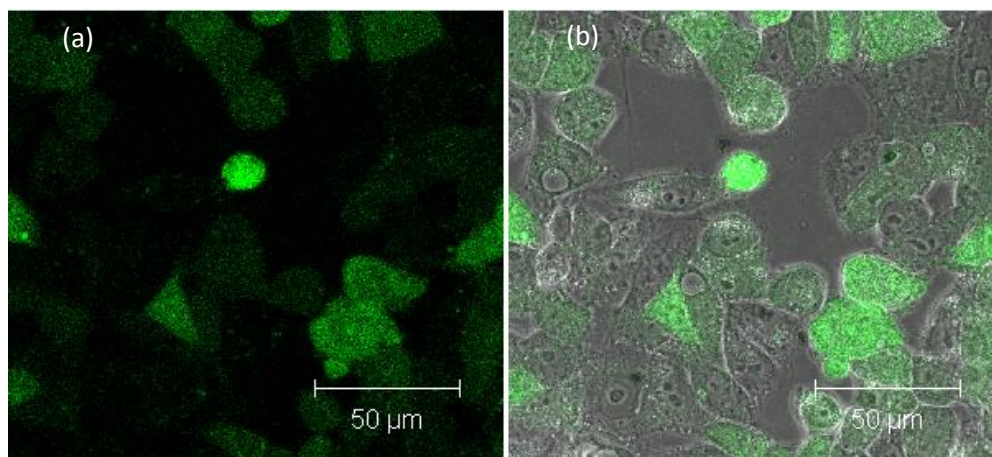


Figure 5.3 Confocal image (x40) of cells exposed to 31.25 μ g/ml AgNP suspended in cell culture media. Image taken on a Zeiss 510 LSM confocal microscope with an argon ion laser, excitation 488nm using a band pass filter 505-530nm to detect DCF. (a) LSM image demonstrating intracellular ROS, (b) overlay of LSM and bright field image.

Figures 5.4 and 5.5 show the toxic response of AgNP to HepG-2 cells, but in the presence of UDCA. The same concentrationdependent response was noted as with the previous assays, following exposure to AgNP alone with this response increasing over time. The MTT assay (figure 5.4) showed a significant decline in cell viability after 96 hours at a concentration of 7.81 μ g/ml. At a 15.6 μ g/ml concentration of AgNP a significant decrease in viability was observed following 48, 72 and 96 hour exposures. It was only at concentrations of 31.25 μ g/ml and above that a significant reduction in cell survival was noted at all exposure time points. Interestingly the presence of UDCA appeared to have had no effect on cellular response to AgNP exposure, with almost identical patterns of toxicity observed both with and without the addition of UDCA. This suggests that the presence of UDCA does not modify AgNP toxicity.

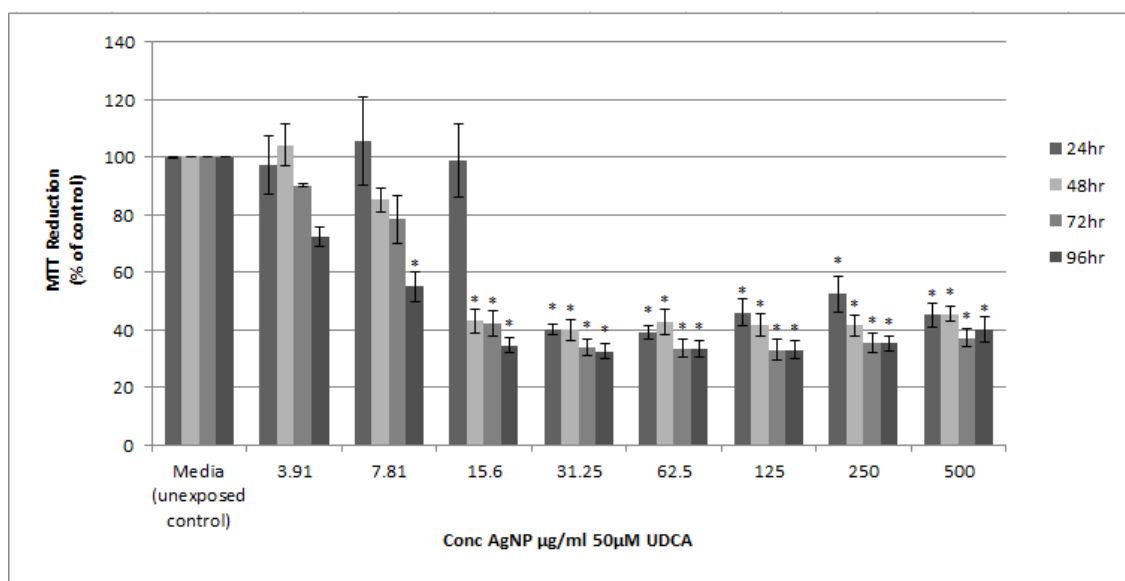


Figure 5.4 Cytotoxicity of AgNP in HepG-2 cells with added UDCA after 24, 48, 72 and 96hr exposures as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

In the presence of UDCA (figure 5.5) a similar pattern of ROS production was observed as with the AgNP exposure alone although it appears more erratic with larger error bars. The same concentration and time dependent response was observed with ROS induction detected following 15 minute exposure at concentrations as low as 3.91 µg/ml. A similar trend was observed with ROS induction in the presence of AgNP alone and a maximum ROS production was observed at a concentration of 500 µg/ml after 12 hour exposure. Following 24 hour exposure ROS production was shown to decline, again this can be attributed to cell death. ROS induction by a concentration of 31.25 µg/ml AgNP was confirmed by confocal microscopy illustrated in figure 5.6. It is apparent from the data that UDCA does not alter oxidative stress induced by AgNP exposure and can be concluded that UDCA does not modify AgNP ability to induce intracellular ROS production.

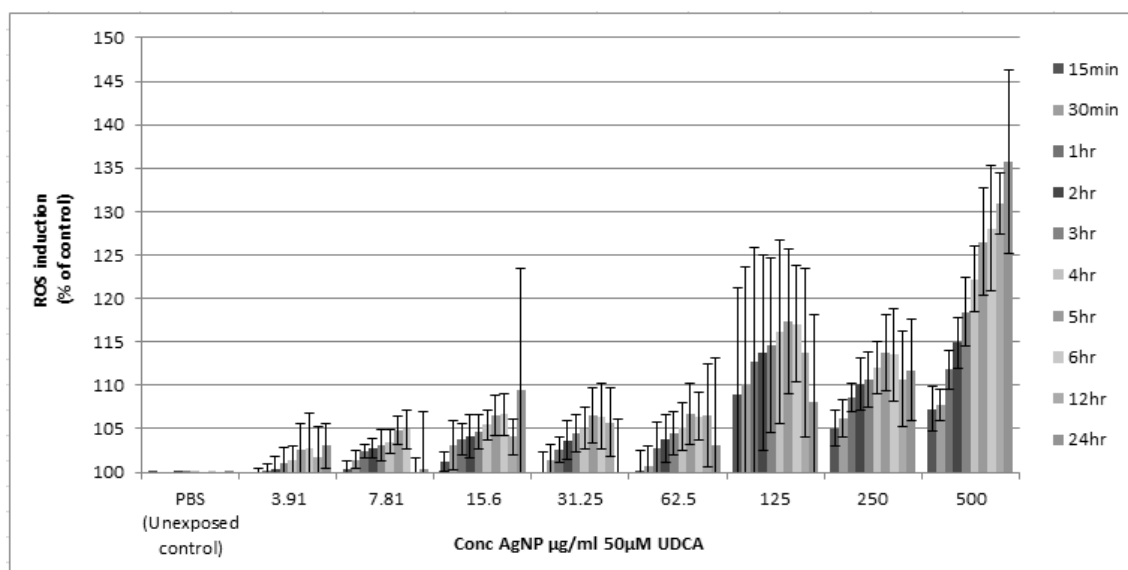


Figure 5.5 ROS generation in HepG-2 cells after different time points of AgNP exposure in the presence of UDCA. Data expressed as percentage of control mean \pm SD of six independent experiments.

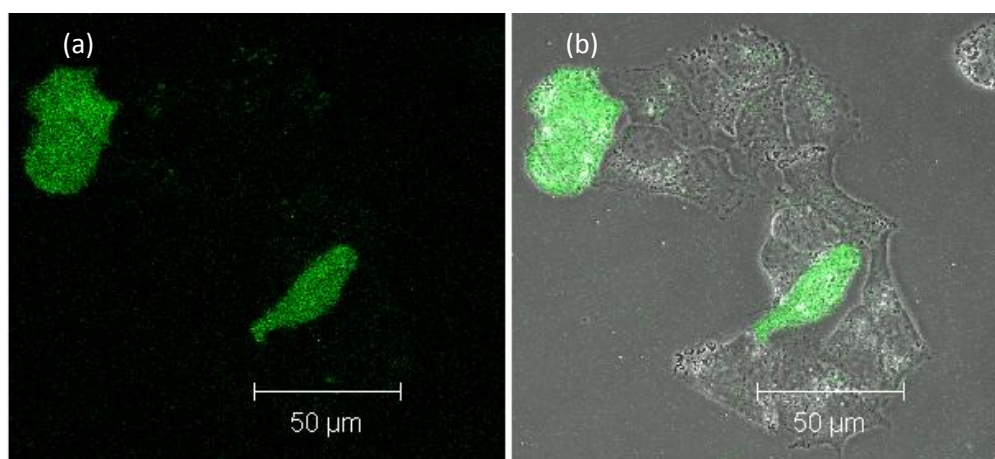


Figure 5.6 Confocal image (x40) of cells exposed to 31.25 µg/ml AgNP suspended in cell culture media in the presence of UDCA. Image taken on a Zeiss 510 LSM confocal microscope with an argon ion laser, excitation 488nm using a band pass filter 505-530nm to detect DCF. (a) LSM image demonstrating intracellular ROS, (b) overlay of LSM and bright field image.

Figure 5.7 represents the exposure of HepG-2 cells to AgNP in the presence of CA. Again a concentration and time dependent response to AgNP exposure was observed with this assay. A noted decline in cell viability can be seen after 24 hours of exposure at an AgNP concentration of 3.91 µg/ml and then an apparent increase in viability compared to the unexposed control after longer exposure periods at the same concentration, indicative of possible cell recovery. At an AgNP concentration of 3.91 µg/ml after a 24 hour exposure a significant decline in cell viability was apparent. At exposure times of 48 and 72 hours a significant decline in viability was also observed when compared to the unexposed control. At 15.6 µg/ml and above, significant reduction in cell survival could be seen at all exposure concentrations when compared to the unexposed negative control. As observed with UDCA, the addition of CA does not appear to modify the toxicity of AgNP and the response in the presence of CA is seemingly identical, with no significant difference or change between the two.

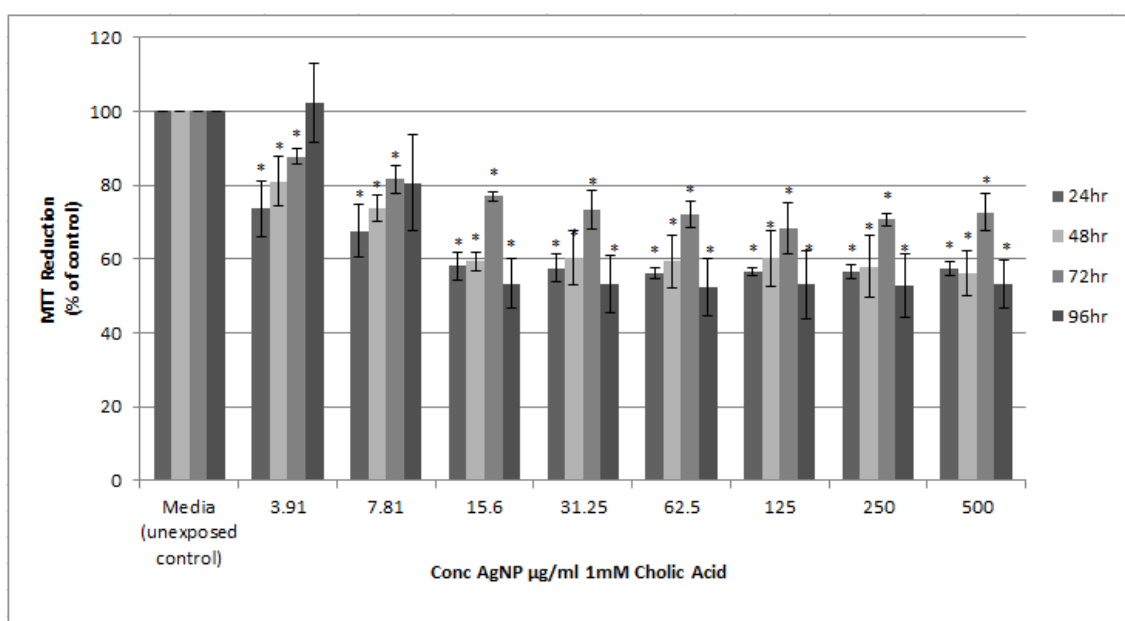


Figure 5.7 Cytotoxicity of AgNP in HepG-2 cells with added CA after 24, 48, 72 and 96hr exposures as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Further studies were performed with the DCF-DA plate assay to determine the ability of AgNP with addition of CA to induce oxidative stress. Figure 5.8 demonstrates ROS production in a concentration and time dependent manner. Generation of ROS was noted at the higher concentrations of 125, 250 and 500 µg/ml. Induction of intracellular ROS was noted after 15 minutes of exposure compared to the unexposed control, with the highest generation detected after this time and at the highest AgNP concentration, 500 µg/ml. After 15 minutes of exposure ROS production begins to decline in a step-wise pattern as exposure time progresses with no ROS detected after the 4 hour exposure point. In comparison to HepG-2 exposure to AgNP alone, the addition of CA alters the pattern of ROS production. In the presence of CA, ROS induction occurs almost immediately after exposure to AgNP and then declines rapidly compared to earlier time points with no ROS detected after 4 hours. Induction of ROS only occurs at the higher exposure concentrations 125, 250 and 500 µg/ml. Exposure to AgNP alone results in a much lower percentage of ROS induction compared to the unexposed control that increases over time and only begins to decline after 12 hours of exposure. In the presence of CA induction of ROS occurs much quicker but declines at a similar rate whereas in the presence of AgNP only, ROS production is more sustained over time. Confirmation of ROS induction by AgNP at a concentration of 3.91 µg/ml in the presence of 1mM CA was verified by confocal microscopy as illustrated in figure 5.9.

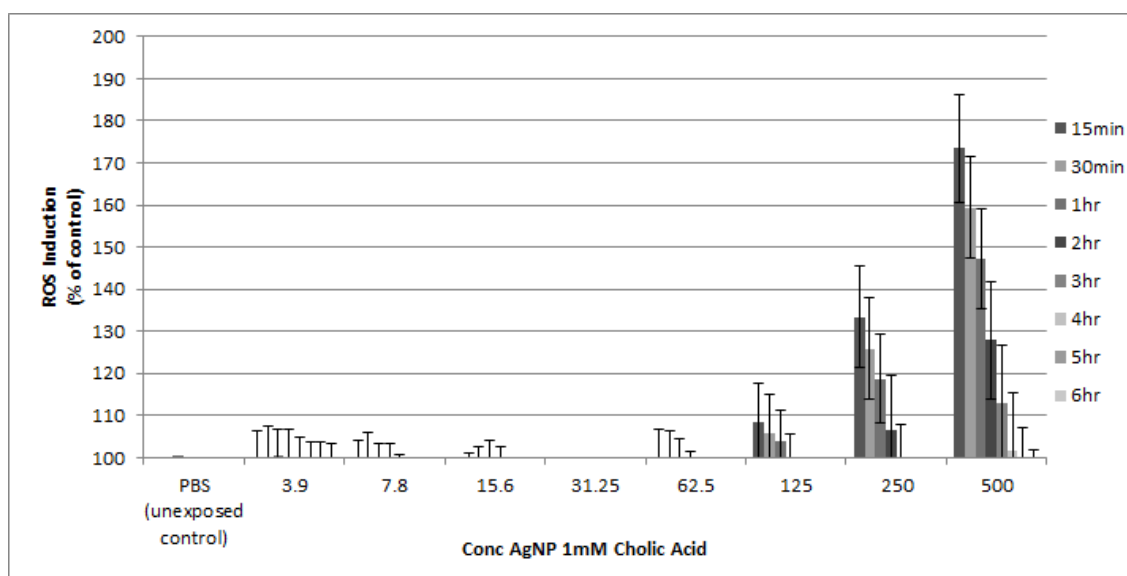


Figure 5.8 ROS generation in HepG-2 cells after different time points of AgNP exposure in the presence of CA. Data expressed as percentage of control mean \pm SD of six independent experiments.

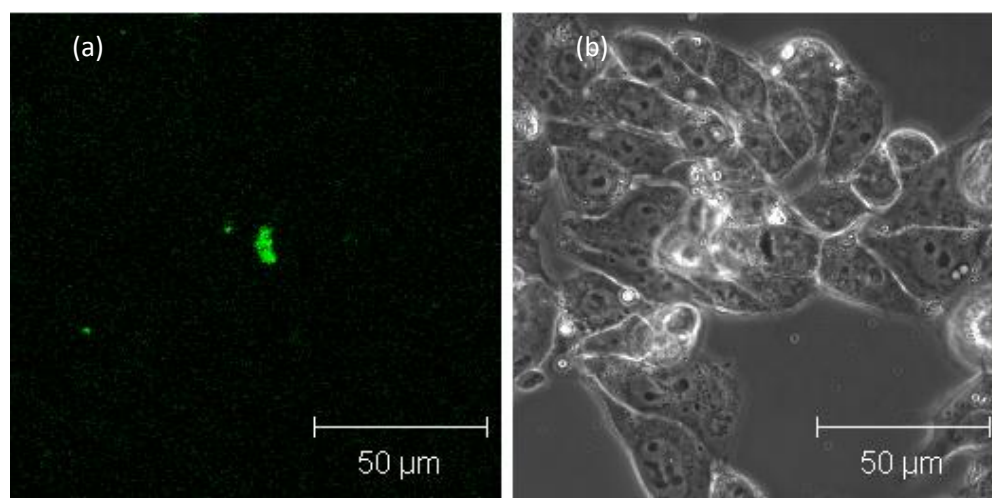


Figure 5.9 Confocal image (x40) of cells exposed to 31.25 μ g/ml AgNP suspended in cell culture media in the presence of CA. Image taken on a Zeiss 510 LSM confocal microscope with an argon ion laser, excitation 488nm using a band pass filter 505-530nm to detect DCF. (a) LSM image demonstrating intracellular ROS, (b) overlay of LSM and bright field image.

Figure 5.10 illustrates the toxic response of HepG-2 cells to AgNP exposure in the presence of DCA. As can be seen in figure 5.10 a loss in mitochondrial integrity is demonstrated at the lowest AgNP concentration of 3.91µg/ml at all exposure time points. Significant loss was noted at a concentration 3.91µg/ml after 24 hour exposure. There was a significant reduction at this concentration after 72 and 96 hour incubations but not after 48 hour incubation again this may be due to cell recovery. As the AgNP are in low doses it is possible they have fallen out of solution at this time and any effect was induced within the first 6-12 hours following exposure. After this point any surviving cells may be starting to recover and replicate. The same pattern is observed at a concentration of 7.81µg/ml. A significant reduction was observed at all exposure time points from a concentration of 15.6µg/ml and above.

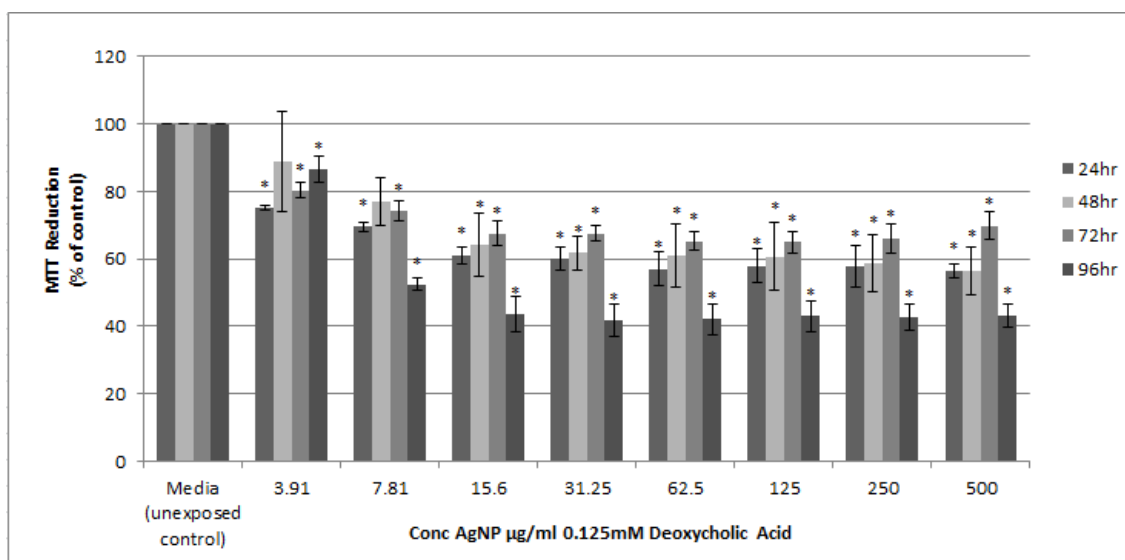


Figure 5.10 Cytotoxicity of AgNP in HepG-2 cells with added DCA after 24, 48, 72 and 96hr exposures as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

The induction of ROS upon exposure to AgNP with the addition of DCA (figure 5.11) follows a similar pattern to that observed with the addition of CA. However it can be seen that ROS are induced at much lower concentrations of AgNP when compared to the CA and in the presence of AgNP alone. ROS were detected after 15 minutes at a

concentration of 31.25 μ g/ml and higher with maximum ROS production occurring at 500 μ g/ml after a 15 minute exposure. After 15 minutes ROS levels began to decline with increasing exposure time. When compared to exposure of HepG-2 cell to AgNP alone, the observed ROS induction occurs at lower concentrations of AgNP and does not increase with time but begins to decline after 15 minutes. Indeed to the case of CA, where ROS induction occurs much earlier but is short lived. It appears that AgNP exposure in the presence of DCA results in the greatest ROS induction compared to the unexposed control and at lower doses when compared to the AgNP exposure with CA and AgNP exposure alone. Confirmation of ROS induction by AgNP at a concentration of 3.91 μ g/ml in the presence of 0.125mM DCA is confirmed by confocal microscopy as illustrated in figure 5.12.

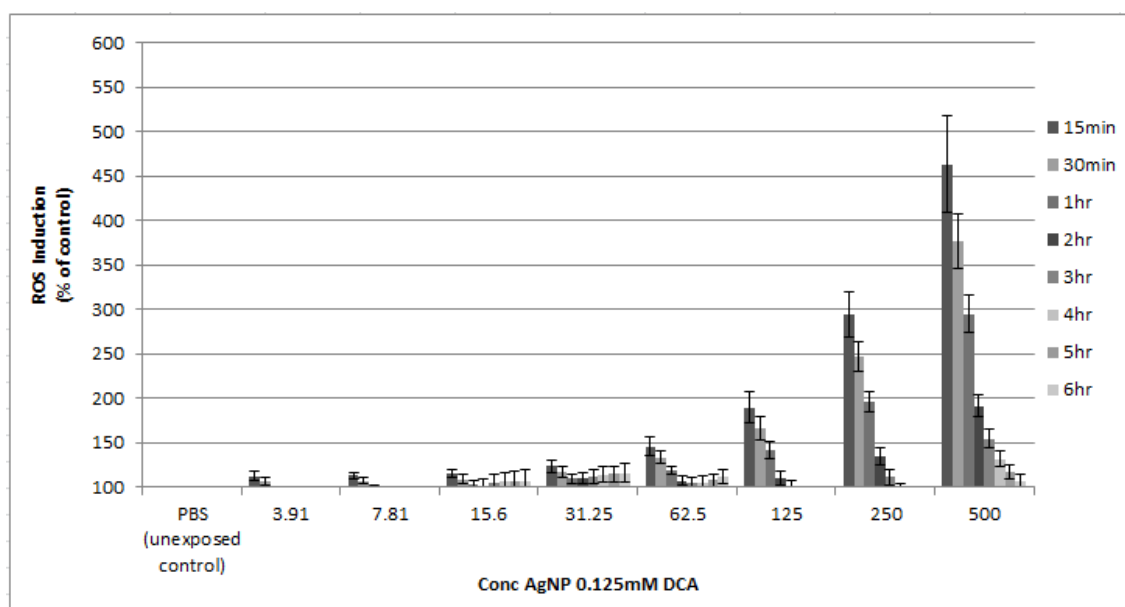


Figure 5.11 ROS generation in HepG-2 cells after different time points of AgNP exposure in the presence of DCA. Data expressed as percentage of control mean \pm SD of six independent experiments.

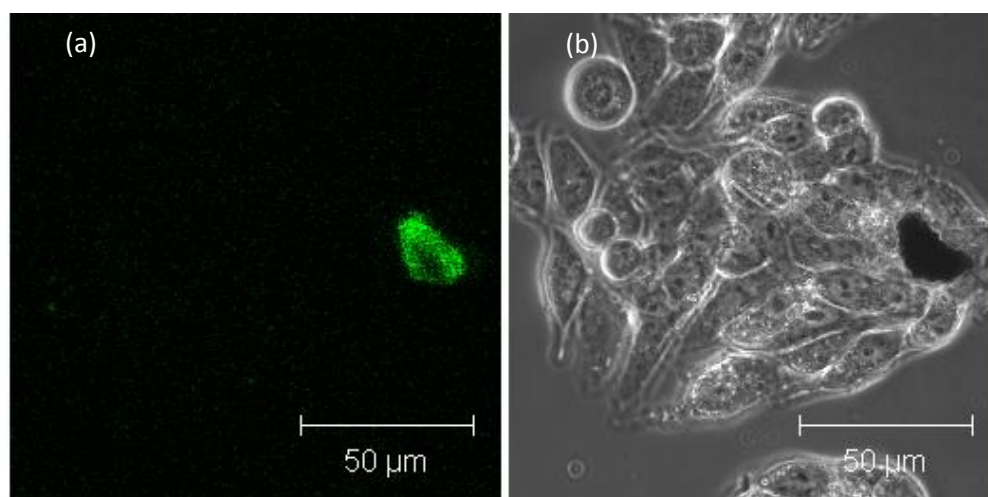


Figure 5.12 Confocal image (x40) of cells exposed to 31.25µg/ml AgNP suspended in cell culture media in the presence of DCA. Image taken on a Zeiss 510 LSM confocal microscope with an argon ion laser, excitation 488nm using a band pass filter 505-530nm to detect DCF. (a) LSM image demonstrating intracellular ROS, (b) overlay of LSM and bright field image.

The AB assay was also performed for the HepG-2 cell line in the presence and absence of UDCA, CA and DCA where a concentration and time dependent response was also observed resulting from AgNP exposure. AB gives a more general picture of the loss in cell viability by targeting the cytosol of the cell and detecting a change in the cytosolic environment. A similar pattern of toxicity was observed with the addition of DCA having a substantial effect on cell viability occurring after 24 hours of exposure (see Appendix A5.2.1 (a)-(d)).

Statistical analysis ($p < 0.05$) comparing the differences in toxicity induced by AgNP alone and in the presence of the tested biofluid components highlighted statistically significant differences between the toxicological data. The presence of CA and DCA significantly altered the cytotoxic effect induced by AgNP on HepG-2 cells, at a concentration of 15.6µg/ml and above the response curves were found to be statistically ($p < 0.05$) different. This change was noted at 48, 72 and 96 hour time points. UDCA was noted not to significantly alter the toxicity of AgNP in HepG-2 cells.

5.2.2 Cytotoxic evaluation of Hep2 cell line to AgNP exposure alone and in the presence of UDCA

As a control, another cell line was tested to establish if biofluid components could modify the effects of AgNP exposure in areas other than their target organs. To investigate this, the Hep2 cell line was selected. The same exposure concentrations were employed and the effects of AgNP toxicity in the presence of biofluid components were investigated. UDCA was selected as the test bile acid. A concentration finding experiment was performed to determine the highest concentration of UDCA that the Hep2 cell line could withstand without causing toxicity. This concentration was found to be 2.2×10^{-7} M and this was the working concentration employed for the following experiments.

Figure 5.13 displays the cytotoxic response of Hep2 cells to AgNP exposure determined by the MTT assay. This assay demonstrates a typical concentration and time dependent pattern with significant reduction in cell survival noted at all concentrations following 72 hour exposure. A significant reduction in viability after 72 and 96 hours was observed at doses of $7.81 \mu\text{g/ml}$ and above and after 48 hours at a concentration of $31.25 \mu\text{g/ml}$ and above. Furthermore a significant decrease in cell survival compared to the unexposed control was observed for all exposure time points at a concentration of $62.5 \mu\text{g/ml}$ and upwards.

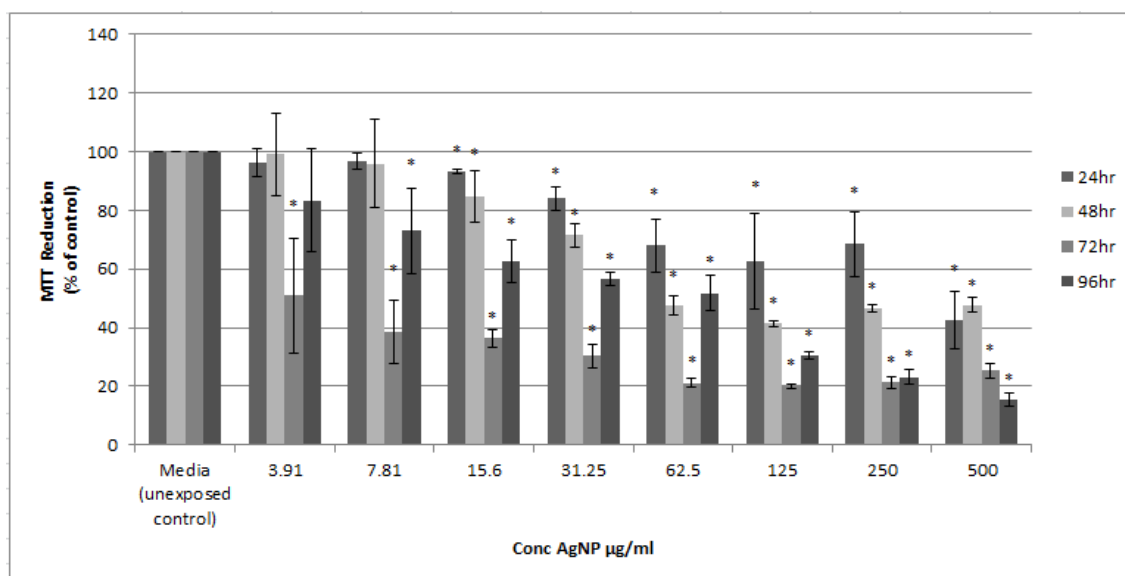


Figure 5.13 Cytotoxicity of AgNP to Hep2 cells after 24, 48, 72 and 96hr exposure as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure 5.14 illustrates the toxic response of AgNP to Hep2 cells in the presence of UDCA. The data demonstrate that in the presence of UDCA there is a dramatic reduction in cell viability compared to when cells are exposed to AgNP alone. No significant change in cell viability occurred after 24 hour exposure but a significant reduction following 48, 72 and 96 hour exposures was noted at all tested concentrations of AgNP. The data presented illustrates that compared to AgNP exposure alone the presence of UDCA does modify toxicity.

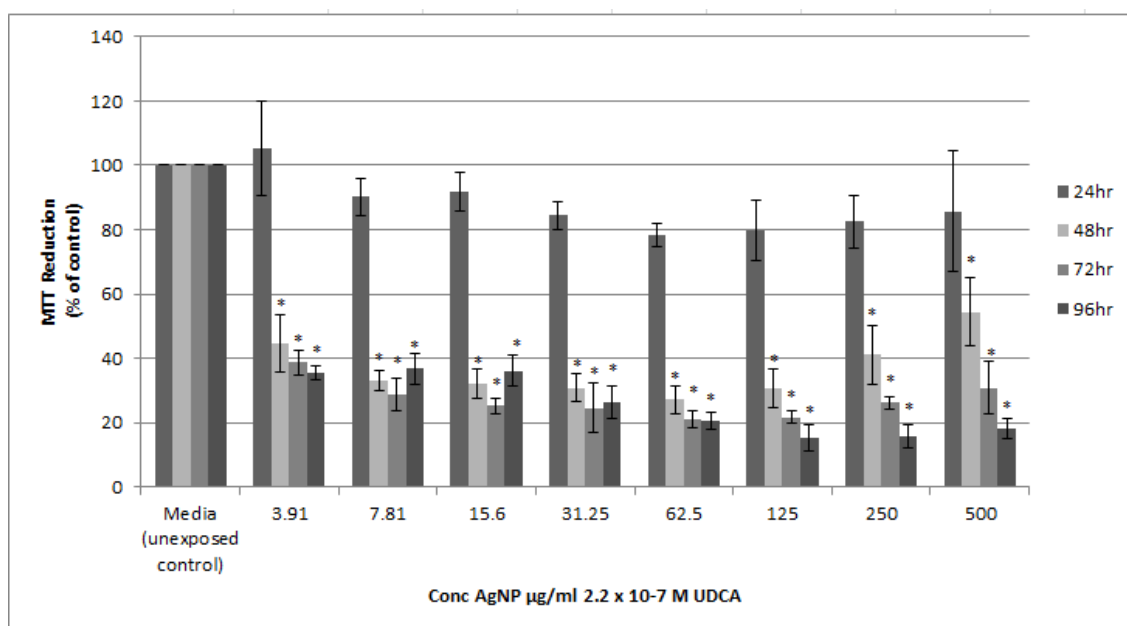


Figure 5.14 Cytotoxicity of AgNP to Hep2 cells with added UDCA after 24, 48, 72 and 96hr exposures as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

A DCF-DA plate assay was also employed to investigate intracellular oxidative stress, to see if the presence of UDCA could influence the generation of ROS in response to AgNP exposure. Exposure to increasing concentrations of AgNP induced ROS production in a concentration and time dependent manner (figure 5.15). ROS production was detected at exposure concentrations of $7.81 \mu\text{g/ml}$ and above with maximum production noted at $500 \mu\text{g/ml}$. Induction of ROS was observed after 15 minutes of exposure with maximum production observed after 1 hour after which levels of detectable ROS began to decline. In the presence of UDCA (figure 5.16) a substantial reduction in ROS generation was observed when compared to exposure to AgNP alone and ROS were only detected at higher concentrations of AgNP, above $250 \mu\text{g/ml}$. A maximum level ROS production with added UDCA was observed at a concentration of $500 \mu\text{g/ml}$ after 1 hour and as illustrated by the data the percentage of ROS induction when compared to the negative control is substantially reduced compared to the amount generated in the absence of UDCA.

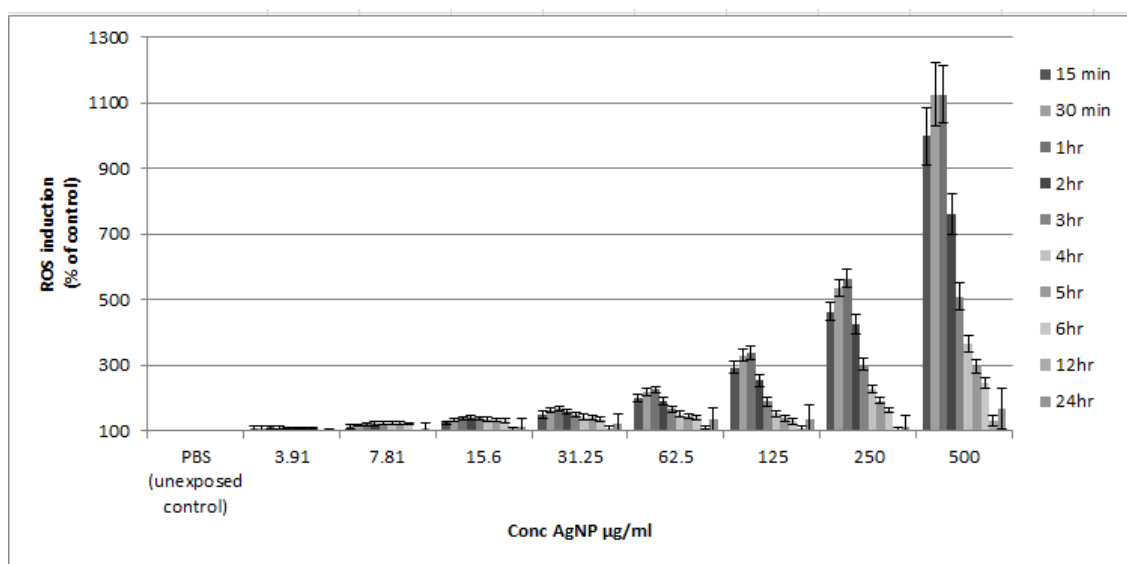


Figure 5.15 ROS generation in Hep2 cells after different time points of AgNP exposure. Data expressed as percentage of control mean \pm SD of six independent experiments

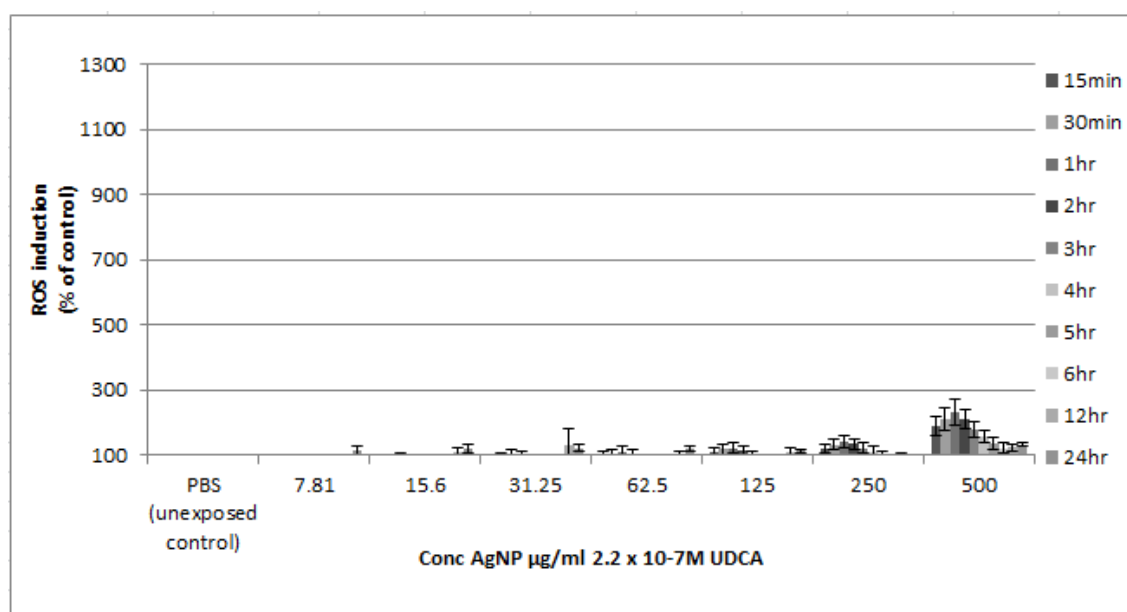


Figure 5.16 ROS generation in Hep2 cells after different time points of AgNP exposure in the presence of UDCA. Data expressed as percentage of control mean \pm SD of six independent experiments

The AB assay was performed for the Hep2 cell line in the presence and absence of UDCA where a concentration and time dependant response was also observed resulting

from AgNP exposure. A similar pattern of toxicity was observed with the addition of UDCA having a substantial effect on cell viability occurring after 24 hours of exposure (see appendix A5.2.2 (a)-(b)).

Statistical analysis ($p < 0.05$) comparing the differences in toxicity induced by AgNP alone and in the presence of UDCA in Hep2 cells highlighted statistically significant differences between the toxicological data. A significant effect on toxicity was noted in the presence of UDCA from the lowest test concentration up to 250 µg/ml after 48 and 96 hour time points compared with exposure to AgNP alone.

5.3 Discussion

It is clear from the literature that exposure to nanoparticles is becoming more widespread mainly due to their continued incorporation into various consumer products. Upon exposure, nanoparticle contact with surfactants and biofluids is inevitable and it is important to take these events into account when examining toxicity. This study aimed to investigate this interaction to identify a possible modification of toxicity by individual components of one of the major biofluids within the body.

As discussed in chapter 4, the physicochemical characteristics of AgNP play an important role in how they will interact with biological systems. The size, associated surface area, shape and chemical composition have been shown to be of great importance to nanoparticle toxicity (Renwick *et al*, 2004; Rai *et al*, 2009; Kam *et al*, 2004). Together with the ion release associated with nanoparticle biofluid interaction, as previously discussed, a number of contributing factors relating to the physicochemical characteristics must be considered when interpreting overall nanoparticle toxicity (Warheit, 2007; Beer *et al*, 2012).

The cytotoxicity studies undertaken illustrate that mitochondrial integrity (MTT) and the general health of the cell (AB) is affected by exposure to AgNP and that the chosen biofluid components have different modifying effects. Table 5.1 concisely illustrates IC₅₀ values for both assays providing an overall synopsis of toxicity. From the data presented it can be suggested CA and DCA do have some modifying effects. While not altering the pattern of toxicity in the viability assays, the pattern of ROS induction is altered upon their addition. The addition of CA and DCA induces greater levels of ROS

induction with DCA producing the greatest response following a 15 minute exposure at the highest concentration of AgNP compared to the unexposed control. AgNP exposure alone produces much lower levels of ROS with maximum values only slight higher compared to the unexposed control. This increase in ROS production and at earlier points of exposure may suggest a more rapid or alternative pathway of cell death in the presence of CA and DCA. Previous studies have shown that lipid peroxidation by nanomaterials is a contributing factor to the generation of ROS and the overwhelming of natural antioxidant systems. ZnO is noted as enhancing lipid peroxidation contributing to increased ROS generation while AgNP exposure in rats can cause increased lipid peroxidation and quench naturally occurring antioxidants. It is postulated that the increased levels of ROS following AgNP-CA and AgNP-DCA exposure enhances lipid peroxidation within cells resulting in the greater levels of ROS noted compared to AgNP exposure alone (Premanathan *et al*, 2011; Adeyemi & Faniyan, 2014). This upsurge in generation may be the result of a reactive product formed by the interaction of AgNP and biofluid components. Further investigation into the mechanism of cell death and the potential role of lipid peroxidation within cells must be investigated.

Table 5.1 Calculated IC₅₀ values (µg/ml) resulting from exposure to AgNP for the AB and MTT assays in the HepG-2 and Hep2 cell lines. * Denotes lethal concentration less than the lowest exposure concentration. IC₅₀ values were calculated from the average response of three independent experiments fitted to a sigmoidal curve and a four parameter logistic model used to calculate Inhibitory Concentration (IC₅₀) with a (p<0.05)

Cell Line	AB assay IC ₅₀ (µg/ml)				MTT assay IC ₅₀ (µg/ml)			
	24hr	48hr	72hr	96hr	24hr	48hr	72hr	96hr
HepG-2	5.50	7.80	5.50	4.20	119.51	45.34	44.59	49.20
HepG-2 + UDCA	6.30	9.10	5.30	5.70	135.06	66.52	31.81	10.97
HepG-2 + CA	4.03	6.24	4.78	7.94	0.0004	0.0003	0.001	215.48
HepG-2 + DCA	4.28	6.49	2.99	6.24	1389.30	746.29	0.002	0.003
Hep2	45.06	31.58	8.80	91.90	388.39	157	2.19	43.41
Hep2 + UDCA	439.16	4.25	0.27	0.94	0.0007	<3.91*	<3.91*	<3.91*

While no modification in toxicity by UDCA was noted in the HepG-2 cell line, modification was noted in Hep2 cells. A delay in toxicity in the first 24 hours after exposure together with a reduction in ROS production strongly suggests that UDCA can modify Hep2 toxic response to AgNP exposure. While it appears to increase AgNP toxicity, ROS induction is significantly reduced suggestive of an antioxidant effect. This may indicate an alternative mechanism of cell death. These data indicate that UDCA can modify AgNP toxicity in a cell type different from that of its normal target.

While it is clear AgNP cause toxicity, it must be considered how much of this toxicity is attributed to the release of silver ions. A study by Beer *et al* (2012) addressed this phenomenon, identifying to what level the fraction of silver ions in various AgNP suspensions (commercially available and laboratory synthesized) influence toxicity (Beer *et al*, 2012). The study concluded that free silver ions in AgNP preparations do indeed play a considerable role in the overall toxicity of AgNP suspensions. However other studies are in agreement that at lower concentrations of metal ions, the uptake of nanoparticles leads to additional toxicity compared to higher concentrations where it appears the particles do not contribute to further toxicity (Navarro *et al*, 2008; Kim *et al*, 2009). Beer *et al* (2012) postulate that the mechanism of toxicity for AgNP is dissolution of particles in lysosomes, previously observed for copper oxide (CuONP) nanoparticles, possibly due to a protective barrier effect of the plasma membrane but this barrier can be evaded by a Trojan horse mechanism when nanoparticles are taken up by cells (Studer *et al*, 2010; Limbach *et al*, 2007). It must also be considered that sonication of AgNP suspensions and other preparation methods may have contributed to silver ion release and therefore toxicity. For example during sonication the energy released in order to break up large AgNP aggregates is converted to thermal energy which may promote dissolution of silver ions from particles (Liu & Hurt, 2010). From this information it is clear that when investigating AgNP toxicity, the role of both silver ions and the particles themselves must be considered.

In order to monitor the potential modification of the chosen biofluid components on AgNP toxicity in cells found outside of their normal target area, an epithelial laryngeal cell line was chosen. The biofluid components under study have been employed as pharmaceutical agents and medications. UDCA is widely used as a method of gallstone dissolution (Arisawa *et al*, 2009; Lapenna *et al*, 2002). This treatment has numerous trade names, including Actigall™ and BILIVER™, and is an oral treatment presented

as an alternative to surgery. CA and DCA also have medical applications. CA is used as treatment for children and adults with inborn errors in bile acid synthesis. Orphacol™ as it is known is taken orally and is vital in preventing the development of cholestatic liver disease (European Medicines Agency, 2013). DCA also has uses as a therapeutic drug used to treat localized fat deposits via subcutaneous injection. Studies have also reported the conjugation of heparin to deoxycholic acid in order to develop an oral delivery system for anticoagulant treatment preferable to the standard intravenous and subcutaneous injection (Lee *et al*, 2001; Kim & Vaishali, 2006). The fact that these bile acids are or may be utilized in a medicinal capacity highlights that they will be present in areas of the body other than the liver. As such the data presented here on a laryngeal cell line is a relevant line of investigation given that in most cases these bile acid derived treatments are given orally. As the data demonstrate, the effect on toxicity in cell lines of different origins must be seriously considered and a more complete cytotoxic profile of AgNP to include exposure site contributions be established.

5.4 Chapter Summary

This chapter has demonstrated the modifying effect of some of the common biofluid components on AgNP toxicity. The data presented demonstrate that biofluid components can significantly influence the toxic profile of AgNP. As such future studies must consider the effects various biofluids and their components have on the processing of nanoparticles upon entry to the body, their presentation to cells, distribution and the biological processes they induce. Finally researchers must not just consider the toxicity of the isolated nanoparticles but that of a combined effect including, the local environment and the ions they release which have been shown to contribute considerably to toxicity.

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Chapter 6

Adapted from “The surfactant dipalmitoylphosphatidylcholine modifies acute responses in alveolar carcinoma cells in response to low concentrationsilver nanoparticle exposure”

6.1 Introduction

Nanoparticle exposure via inhalation is probably the most common entry point for nanoparticles and during recent decades the generation of engineered nanoparticles for applications in medicine, food, cosmetics and electronics to name a few has increased the likelihood of exposure exponentially (Sozar & Kokini, 2009; Bouwmeester *et al*, 2009).

Inhalation provides an easy entry route for engineered nanoparticles in nano-carrier systems for drug delivery allowing an easy route for targeted, controlled and non-invasive delivery. Liposomes in particular have been the nano-carrier of choice in the development of improved and intelligent drug delivery systems (Varez-Lorenzo *et al*, 2009; Shum *et al*, 2001). While inhalation provides an alternative method of pharmaceutical delivery, improving both the treatment and comfort of patients, it also provides easy entry for undesirable nanoparticles. A number of studies have been undertaken to explore lung entry of various nanoparticles and their subsequent distribution around the body. Cerium oxide (CeO₂) nanoparticles have been shown to cause acute toxicity in the lungs following exposure. It was postulated that this could lead to chronic inflammation days after the exposure period with particles deposited in the lungs as they penetrate through the alveolar wall into the systemic circulation (Srinivas *et al*, 2010). Silver nanoparticle (AgNP) exposure in the lung has also been shown to induce cytotoxicity along with zinc oxide (ZnO) and titanium dioxide (TiO₂). Although all nanometals induced toxicity they did so at differing levels with ZnO inducing greater levels of toxicity in alveolar macrophages compared with the other two nanometals. The induced toxicity may also lead to excessive secretion of certain biological agents causing dysfunction in the cytokine network, reducing the efficiency of the respiratory immune system (Liu *et al*, 2013).

Due mainly to their antibacterial qualities AgNP are currently one of the most utilized nanomaterials on the market providing a diverse range of applications including medical devices, antibacterial textiles and toys and food packaging (Sozar & Kokini, 2009; Bouwmeester *et al*, 2009). While AgNP have been highly regarded for their benefits, their increased incorporation into everyday consumer products has generated considerable interest in establishing the risk exposure has on human health and the environment. A more in depth toxicological assessment of nanoparticle-biological interaction is required and a focus must now be shifted onto the influence of biofluids and surfactants on toxicological outcome (Mwilu *et al*, 2013; Ehrenberg *et al*, 2009; Wang *et al*, 2013; Misra *et al*, 2012; Aggarwal *et al*, 2009). In light of this and the easy entry of nanoparticles both beneficial and otherwise, this study investigates the interaction and toxicological influence of a component of lung surfactant on AgNP toxicity on the lung.

Pulmonary surfactant is composed of a variety of complex phospholipids, lipids and surfactant specific proteins. Dipalmitoylphosphatidylcholine (DPPC) is the most abundant phospholipid, comprising 70-80% of total surfactant. The overall function is to reduce surface tension at the liquid-air interface at the bronchoalveolar surface with a dysfunction in surfactant associated with certain respiratory disorders (Kumar & Bohidar, 2010; Serrano & Perez-Gil, 2006; Bakshi *et al*, 2008). In recent studies DPPC has been used to improve the properties of liposomes to produce more successful *in vivo* methods of delivery. For example its inclusion in liposome encapsulation of cancer drugs can improve delivery of the complete drug concentration to tumour cells improving killing of cancer cells (Yavlovich *et al*, 2011).

The aim of this investigation is to assess the toxic response of a carcinogenic lung cell line, A549, to *in vitro* exposure to AgNP. The ability of DPPC to affect or modify any observed toxicity was also established. The physicochemical characteristics of nanoparticles play a vital role in toxicological investigation and were previously discussed in chapter 4 together with the effect of DPPC on their characteristics. A cytotoxic profile was carried out using a number of viability assays including 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), alamar blue and a 2', 7'-dichlorofluorescein diacetate (DCFH-DA) plate assay to detect intracellular oxidative stress induced by exposure. The influence of DPPC on the outcome of toxicity was also assessed using the same assays. A subsequent study was performed to monitor the effect

on the release of inflammatory markers interleukin-8 (IL-8) and tumour necrosis factor alpha (TNF- α) upon exposure to AgNP in the presence of DPPC.

6.2 Cytotoxicity Testing

The cytotoxicity of AgNP on A549 cells was assessed using the viability assays MTT, Alamar blue (AB) and a DCF-DA plate assay to detect intracellular oxidative stress. The influence of DPPC on the observed toxicity was investigated using the same array of toxicological assays. A positive control of 10% DMSO was employed for all viability assays and 5 μ M H₂O₂ was employed for ROS studies. The ability of AgNP to induce an inflammatory response and the impact of DPPC on this response was also investigated using ELISA based assays for the detection of IL-8 and TNF- α release. Cytotoxicity data for AgNP exposure is presented in table 6.1. The results of the MTT assay are presented for toxicity testing in the presence and absence of DPPC. Both MTT and AB assays were performed. Results for AB assay are presented in Appendix A6.2.

6.2.1 Cytotoxic evaluation A549 cell line to AgNP exposure alone and in the presence of DPPC

Figure 6.1 represents the cytotoxic response of A549 cells to AgNP exposure as determined by the MTT assay. Reduction in cell viability due to exposure occurred in a concentration and time dependent manner. A significant reduction in mitochondrial integrity was observed after 48 hours at all AgNP concentrations. Significant reduction after 72 and 96 hour exposures was noted at concentrations of 15.6 μ g/ml and above compared to the unexposed control, while a significant reduction in cell survival at all exposure times occurred at 31.25 μ g/ml and above.

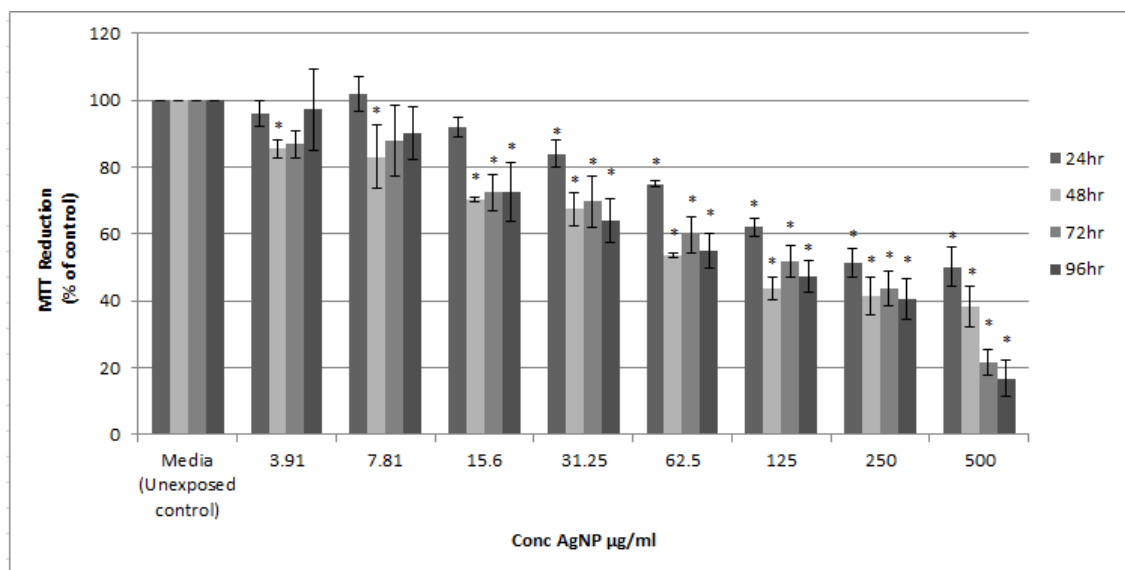


Figure 6.1 Cytotoxicity of AgNP in A549 cells after 24, 48, 72 and 96hr exposure as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

A DCF-DA plate assay was employed to detect any ROS production in response to AgNP exposure over a 6 hour period with readings taken every hour. This study indicated no intracellular ROS production in response to AgNP exposure. The generation of intracellular hydro peroxides was noted at the highest concentration, 500µg/ml, with production increasing in a time dependant manner. Induction of ROS was first detected at this concentration after 15 minutes, with the highest induction observed after 1 hour. Results are presented in Appendix A 6.2.

The ability of DPPC to influence toxicity was investigated using the same array of cytotoxicological tests.

Figure 6.2 displays the toxic response of A549 cells to AgNP in the presence of 0.25µg/ml DPPC. A concentration dependant response to AgNP exposure was observed. A change in toxicity was not noted, however the pattern appeared altered in the presence of DPPC. A short delay in toxicity between 48 and 72 hours was observed after which a significant reduction in cell viability occurred. Significant reduction in cell survival to all exposure concentrations was noted after 72 hours. After 24 hours, significant loss in cell viability was observed at a concentration of 15.6µg/ml with a

significant decline at 7.8 μ g/ml and above following 96 hour exposure. Significant cell death compared to the unexposed control at all exposure intervals was observed at 62.5 μ g/ml and above. The addition of DPPC may alter the pattern of toxicity by causing a delay in the first 48 hours, after which a significant reduction in viability is observed at 72 hours.

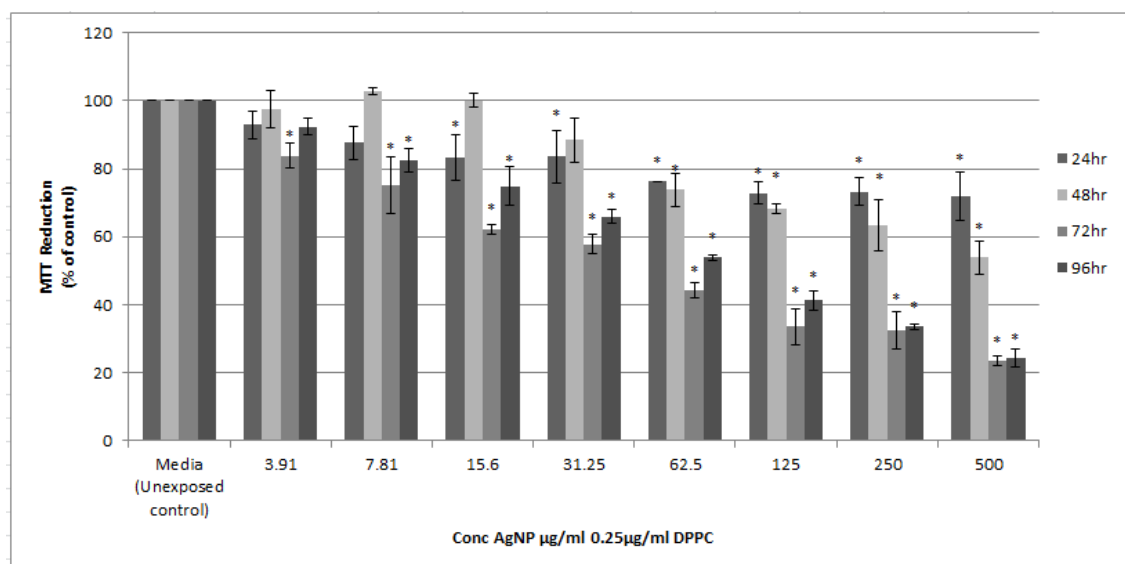


Figure 6.2 Cytotoxicity of AgNP in A549 cells with added DPPC after 24, 48, 72 and 96 hour exposures as determined by the MTT assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

The delay and increase in viability at lower nanoparticle concentrations at 48 hours may result from surviving cells beginning to replicate. DPPC is a surfactant and as such will associate with the nanoparticles surface. This association may result in a “Trojan horse” effect or delayed toxicity as observed at lower concentrations in the first 48 hours. After this time it is possible the DPPC coating has been removed following nanoparticle entry to the cell and trafficking to various cellular compartments allowing the naked nanoparticle to exert its toxic effects (Wang *et al*, 2013).

In addition to cytotoxic evaluation, the clonogenic assay was performed to analyse the effect of longer exposures to AgNP. This assay was performed over a 10 day exposure period and monitored the ability of cells to form colonies in the presence of AgNP. The

ability of cells to proliferate following AgNP exposure with the addition of DPPC was significantly reduced compared to the unexposed control. This was observed at all exposure concentrations. It was found that the ability of A549 cells to proliferate was inhibited at the lowest concentration of 3.91 $\mu\text{g/ml}$ and above with no colony formation and therefore no cell survival at concentrations of 62.5 $\mu\text{g/ml}$ and above, as shown in figure 6.3. Interestingly, it appeared that the addition of DPPC increased the toxicity of AgNP towards cells with significant reductions in colony formation compared with exposure to AgNP alone.

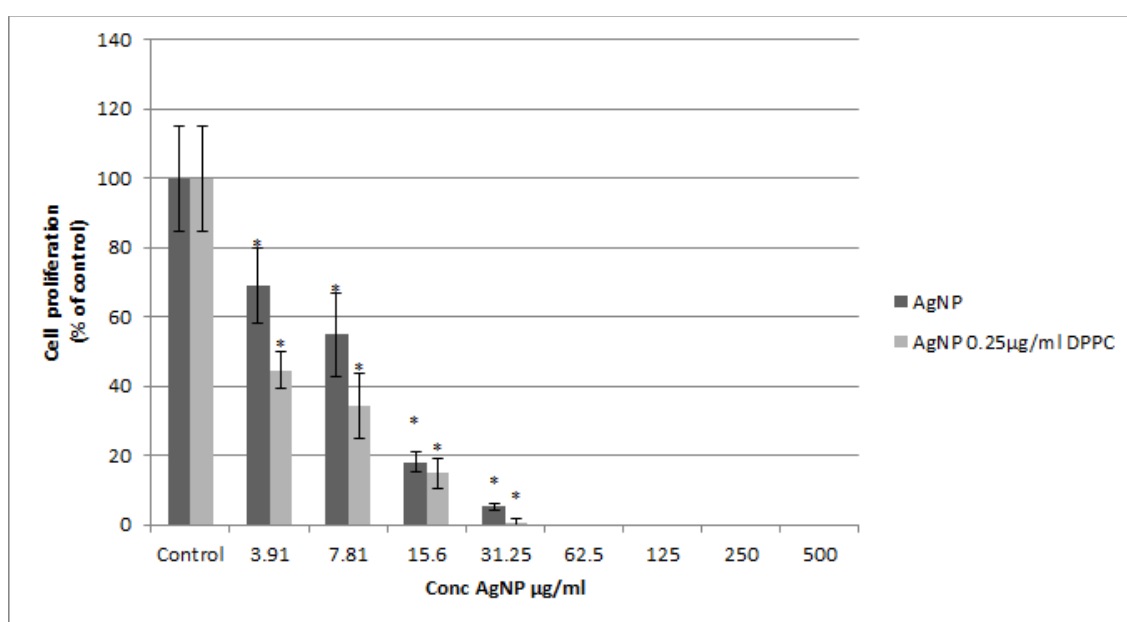


Figure 6.3 Cytotoxicity of AgNP alone and with added DPPC after 10 day exposure as determined by the clonogenic assay. Data are expressed as percentage of control mean \pm SD of four independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

A DCF-DA plate assay was employed to investigate the effect of DPPC on ROS generation in A549 cells following AgNP exposure. From the data it appeared that in the presence of DPPC there was increased generation of intracellular ROS from a concentration of 31.25 $\mu\text{g/ml}$ and above. Figure 6.4 illustrates ROS production in a concentration and time dependant manner. Induction was detected at concentrations as low as 31.25 $\mu\text{g/ml}$ after 15 minutes exposure, with maximum production noted at

500 μ g/ml after 30 minutes. ROS production was detected up to 6 hours after initial exposure at a concentration of 500 μ g/ml and up to 4 hours after initial exposure at 250 μ g/ml. Significant increases were noted at doses of 62.5 μ g/ml after 30 minutes, 125 μ g/ml after 15 and 30 minute exposures and at higher doses significant generation was observed from 15 to 30 minutes and 2 to 3 hours at 250 μ g/ml and at 15 minutes to 5 hours at 500 μ g/ml. From the data it is clear that the presence of DPPC modifies oxidative stress in A549 cells by increasing intracellular ROS formation following AgNP exposure.

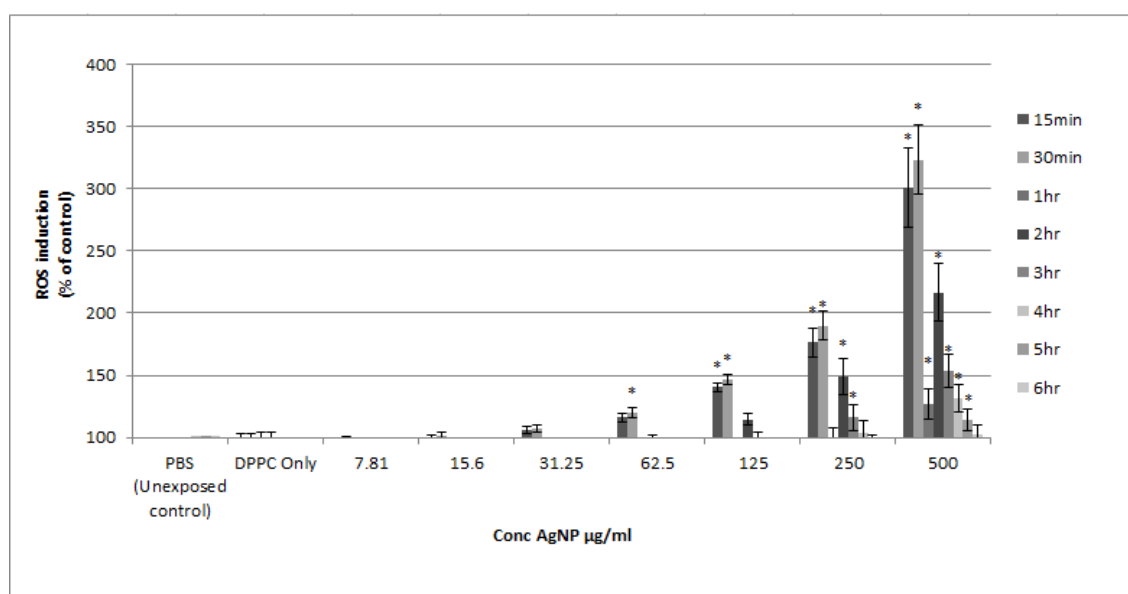


Figure 6.4 ROS generation in A549 cells after different time points of AgNP exposure in the presence of DPPC. Data expressed as percentage of control mean \pm SD of six independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

6.3 Inflammatory Studies

The ability of AgNP to induce IL-8 and TNF- α production and the effect of the addition of DPPC was investigated using ELISA based assays.

Figure 6.5 demonstrates IL-8 release following exposure to AgNP. At the highest concentration, 50 μ g/ml, production of IL-8 was observed after 2 hours. Levels were shown to decline after 3 hours with a second peak in levels demonstrated after 4 hours.

The lower concentration of AgNP caused IL-8 release initially after exposure with increasing generation up to 2 hours after exposure. A similar reduction in levels was detected at 3 hours after which an increase in IL-8 was detected at 4 and 6 hours. A significant increase in IL-8 compared to the unexposed control was noted at 2, 3, 4 and 6 hours at a concentration of 50 μ /ml and at all intervals at 25 μ g/ml. The data suggest that at sub-lethal levels of AgNP, 25 μ g/ml, a more immediate and long lived inflammatory response is initiated compared to higher toxic doses.

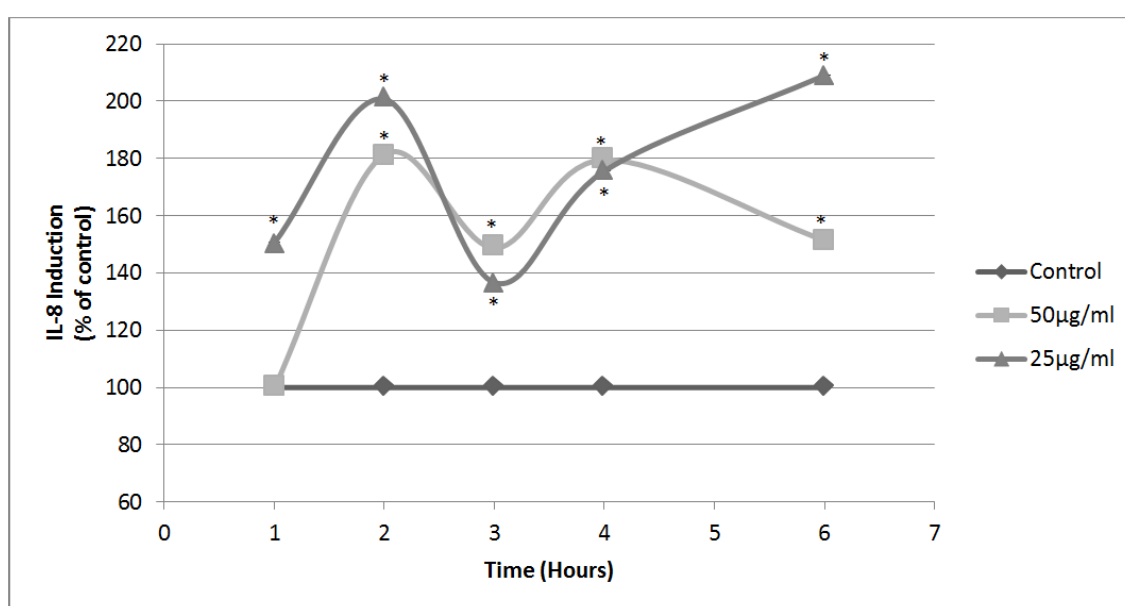


Figure 6.5 Induction of IL-8 in A549 cells at 1-4 and 6 hours following exposure to AgNP. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

Figure 6.6 represents IL-8 release in A549 cells following AgNP exposure with the addition of DPPC. The data show that the presence of DPPC alters the pattern of IL-8 release in cells. The levels of IL-8 detected were substantially higher than exposure to AgNP alone and production occurs at a faster rate with greater levels detected after 1 hour exposure. Similar to figure 6.5 a second spike in release was detected at 6 hours. The presence of DPPC generated an immediate and prolonged inflammatory response compared to exposure to AgNP alone as illustrated by IL-8 levels detected at 1 and 6 hours. Significant release was noted at 1, 2, 3 and 6 hours for all doses compared to the

unexposed control. While DPPC leads to an enhanced production of IL-8, the pattern appears not to be dependent on concentration suggesting that regardless of AgNP concentration the same biological response is initiated.

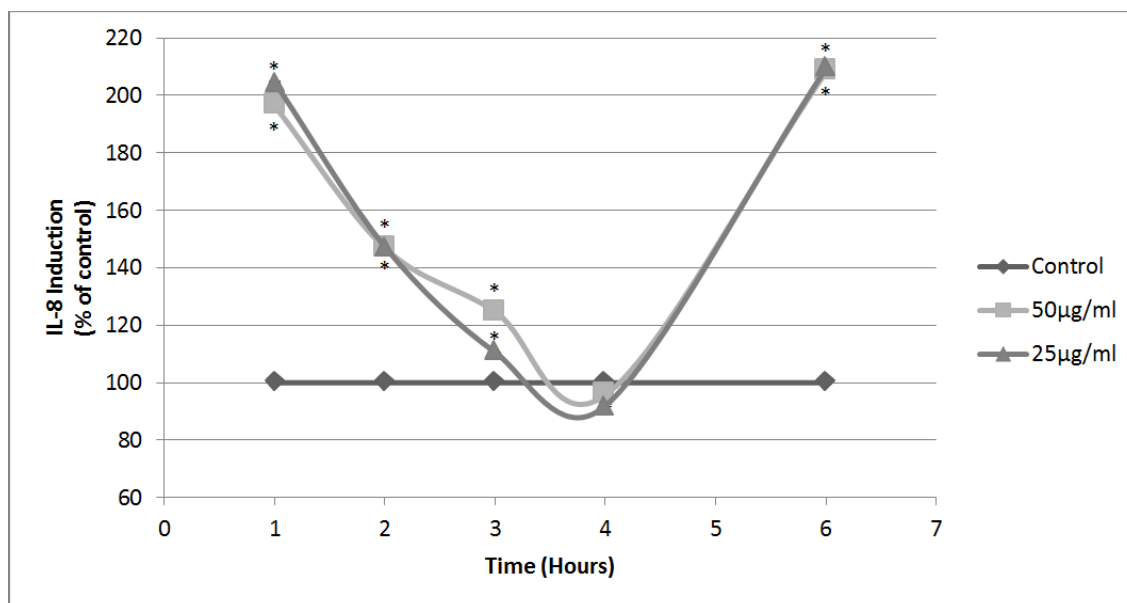


Figure 6.6 Induction of IL-8 in A549 cells at 1-4 and 6 hours following exposure to AgNP in the presence of DPPC. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

Figure 6.7 illustrates TNF- α release following AgNP exposure. Generation of TNF- α at a concentration of 25µg/ml was immediate and short lived with peak levels detected after 1 hour and a rapid reduction observed at 2 hours and above. A similar pattern was detected at the highest concentration with high levels released after 1 hour followed by a swift decline. However as demonstrated with IL-8, a second spike in TNF- α levels occurs at 6 hours. Significant TNF- α release was detected after 1 hour following 25µg/ml exposure and after 1, 2, 3 and 6 hours after 50µg/ml exposure.

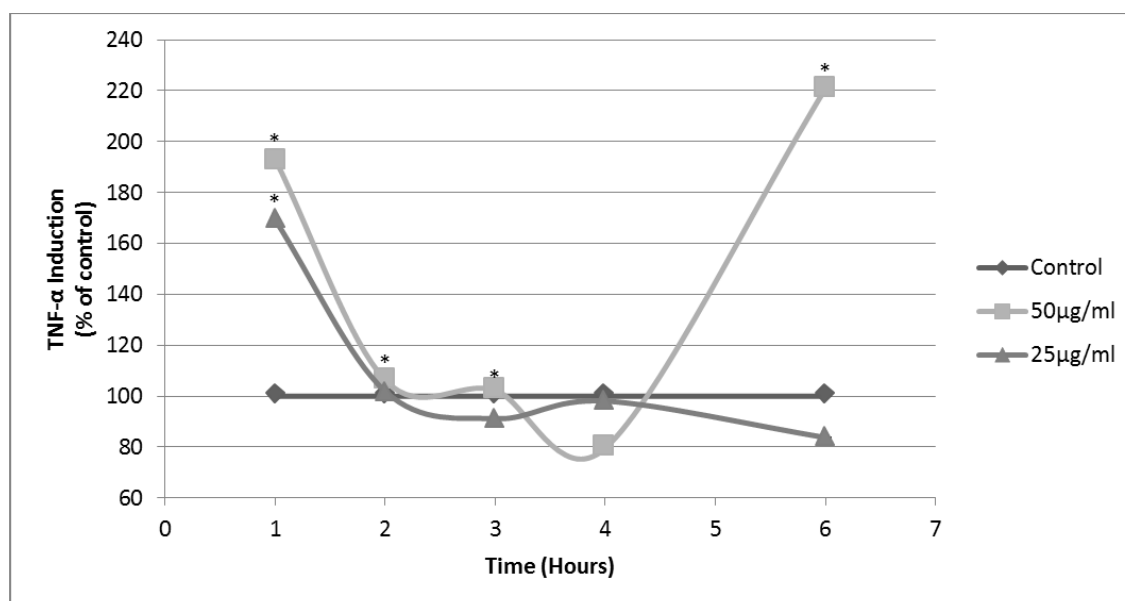


Figure 6.7 Induction of TNF- α in A549 cells at 1-4 and 6 hours following exposure to AgNP. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

In the presence of DPPC (figure 6.8) substantially lower levels of TNF- α were released compared to AgNP alone. TNF- α was generated after 1 hour exposure with a decline in levels detected after at 2 hours. Unlike exposure to AgNP alone, the second increase in levels was detected at 3 hours and gradually increased with time. Significant increases in TNF- α release were noted at 1, 3, 4 and 6 hours at a concentration of 50μg/ml and at 1, 4 and 6 hours at 25μg/ml. It appears the presence of DPPC produces a prolonged inflammatory response similar to that observed with IL-8 release. The addition of DPPC also modifies the pattern of TNF- α release, which similar to IL-8, is not dependent on AgNP concentration.

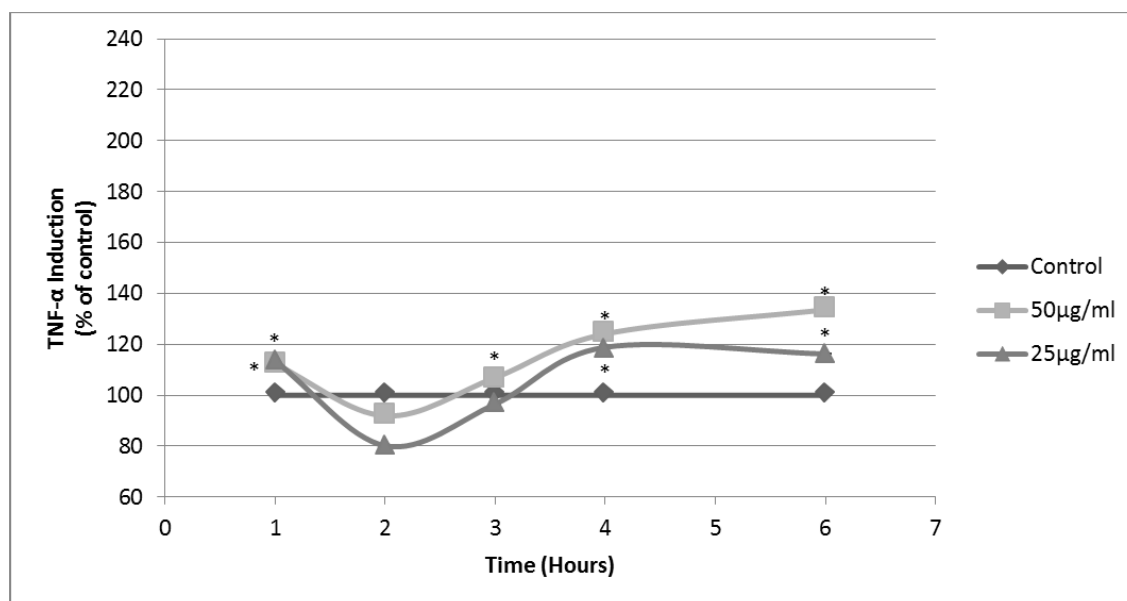


Figure 6.8 Induction of TNF- α in A549 cells at 1-4 and 6 hours following exposure to AgNP in the presence of DPPC. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control

6.4 Discussion

It is apparent from the literature that exposure to nanoparticles is becoming more widespread mainly through their continued incorporation into consumer products. As a result there is an increasing incidence of direct contact between nanoparticles and biological systems. This growing concern of nanoparticle exposure highlights the need for a complete and thorough toxicological investigation to determine what effects are induced and what factors within biological systems play a role in the resulting effects.

The physicochemical characteristics of AgNP play an important role in how they will interact with biological systems. In this instance a thorough characterisation of nanoparticles was undertaken and previously discussed in chapter 4, with no interaction between components evident. Despite the lack of interaction, the effect individual components play on resulting toxicity must always be considered as physicochemical characterisation can never truly represent interactions occurring *in vitro* or *in vivo*.

Table 6.1 displays cytotoxic data for both viability assays. The AB assay demonstrates an increase in IC₅₀ values at 72 and 96 hours. This assay has multiple sites of conversion

within the cell and thus gives an indication into both viability and general cell function. As such the increase in cell viability observed may be attributed to cell recovery. The A549 cell line has a doubling time of 22 hours and it is possible that after the first 48 hours of exposure where toxicity is observed any surviving cells begin to replicate again slowly resulting in an increase in IC₅₀ values. This was also observed at 96 hours with the addition of DPPC. In the MTT assay which is indicative of mitochondrial integrity only, a time dependant reduction in values was observed. Exposure to AgNP was also noted to induce an inflammatory response with release of IL-8 and TNF- α . The presence of DPPC significantly altered the levels of release induced by exposure on A549 cells. The difference in levels released when DPPC was present compared to AgNP exposure alone was found to be statistically ($p < 0.05$) significant at all exposure concentrations and time points.

Table 6.1 Calculated IC₅₀ values ($\mu\text{g/ml}$) resulting from exposure to AgNP for the AB and MTT assays in the A549 cell line. ** Denotes where a 50% effect was not observed. IC₅₀ values were calculated from the average response of three independent experiments fitted to a sigmoidal curve and a four parameter logistic model used to calculate Inhibitory Concentration (IC₅₀) with a ($p < 0.05$)

Cell Line	AB Assay IC ₅₀ ($\mu\text{g/ml}$)				MTT Assay IC ₅₀ ($\mu\text{g/ml}$)			
	24hr	48hr	72hr	96hr	24hr	48hr	72hr	96hr
A549	116.23	11.68	39.86	118.40	340	115	116	91
A549 + DPPC	46.06	45.30	41.78	96.84	**	**	49	80

The mechanism of cell death caused by AgNP can be related to oxidative stress. Mitochondrial cell death by ROS generation has been described in a number of studies as the primary mechanism of nanoparticle related cell death with its generation forming a critical step in initiation of apoptotic pathways (Martindale & Holbrook, 2002; Sastre *et al*, 2000; Piao *et al*, 2011). ROS are generated under normal physiological conditions and are continually eliminated by naturally occurring antioxidants. Under certain “stressful” conditions cells are unable to maintain homeostasis as increased quantities of

ROS are produced which overwhelm the antioxidant systems (Arora *et al*, 2008; Piao *et al*, 2011). Previous studies have shown that AgNP not only induce ROS but can also inhibit antioxidants such as glutathione (Arora *et al*, 2008; Hussain *et al*, 2005; Chairuangkitti *et al*, 2013; Piao *et al*, 2011). This process may be in part due to lipid peroxidation caused by nanoparticle exposure. AgNP and ZnO have been identified as a cause of lipid peroxidation enhancing ROS production while also quenching naturally occurring antioxidants (Premanathan *et al*, 2011; Adeyemi & Faniyan, 2014). This response induces downstream dysfunction such as inflammation and DNA damage within the cell, culminating in cell death (Nel *et al*, 2006). While mitochondrial involvement appears to be the mechanism of cell death, other pathways have been hypothesised. Death via a ROS-independent pathway has also been observed in A549 cells following exposure to AgNP. This results in cell cycle arrest rather than cell death by interfering with a regulatory protein critical in progression of the cell cycle (Chairuangkitti *et al*, 2013). This may partially explain the lack of ROS induction observed following AgNP exposure but with a reduction in viability still evident. Cells may be arrested at various stages of the cell cycle without the involvement of ROS following AgNP exposure as demonstrated in the data.

The induction of oxidative stress is a critical step in the biological response to nanoparticle exposure and causes a number of cellular implications including an enhanced inflammatory response. Excessive ROS production leads to activation of a number of pro-inflammatory cytokines including IL-8 and TNF- α within the lung, which have been implicated in certain respiratory disorders (Henricks & Nijkamp, 2001; Zuo *et al*, 2013). IL-8 is involved in the activation and migration of cells such as neutrophils, during acute inflammation. It can also up-regulate adhesion molecules involved in adherence and migration of cells through the endothelium (Feghali & Wright, 1997; Lim *et al*, 2012). The lung epithelium in particular is an important source of IL-8 and as such is a key player in the mediation of lung inflammation (Donaldson *et al*, 1998). IL-8 has been the focus of a number of studies related to nanoparticle toxicity, with its release implicated in the recruitment of inflammatory cells to the lung (Singh *et al*, 2007). Specifically, following AgNP exposure, significant release of IL-8 from macrophages has been demonstrated, with both particle size and the generation of ROS playing a crucial role in its release (Lim *et al*, 2012; Park *et al*, 2011).

TNF- α is a potent cytokine involved in the initiation and maintenance of inflammation (Nathan, 2002). TNF- α can mediate a number of responses directly such as fever and, tissue damage, as well as causing indirect effects by downstream signalling leading to the activation of other inflammatory factors including IL-6 (Feghali & Wright, 1997; Warren, 1990). This cytokine has been implicated in the development of pulmonary fibrosis following silica micro-particle exposure causing increased endothelial permeability to macromolecules as well as its up-regulation following exposure to various nanoparticles (Napierska *et al*, 2012; Henning *et al*, 1987; Pryhuber *et al*, 2003; Piguet *et al*, 1990). The detection of increased levels of both IL-8 and TNF- α following exposure implicates AgNP in the inflammatory process with an augmented response observed following the addition of DPPC. The increased levels of IL-8 in particular correlates to the ROS generation observed following its addition. In the case of IL-8, an immediate release is observed in the presence of DPPC and its early release has been proposed as a potential bio-marker for acute inflammation in relation to AgNP exposure which the data presented here supports (Lim *et al*, 2012; Park *et al*, 2011).

The ability of the DPPC to increase and prolong the activation of both cytokines compared to AgNP alone suggests progression from acute to chronic inflammation. It has been proposed that high levels coupled with sustained release of these cytokines may prevent resolution resulting in the development of chronic inflammation. It was observed that in certain cases it was not the highest AgNP concentration that generated the greatest cytokine release. This is not surprising as high concentrations can cause cells to enter a death process whereas lower concentrations can induce an inflammatory reaction. Lim *et al* demonstrated cytokine gene expression at sub lethal concentrations of AgNP, in particular IL-8 release at 2.5 μ g/ml (Lim *et al*, 2012).

DPPC is a major component of lung surfactant produced by type II alveolar cells, similar to A549 cells, and as such has been used as a surfactant model in numerous toxicity studies (Kumar & Bohidar, 2010; Dobbs *et al*, 1987; Foucaud *et al*, 2007; Porter *et al*, 2008; Sager *et al*, 2007). Employed as a particle coating, DPPC has been shown to exert a protective effect preventing degradation and allowing controlled release of encapsulated drug compounds; however there is increasing evidence of its contribution to toxicity and induction of oxidative stress (Kaviratna & Banerjee, 2009; Herzog *et al*, 2009; Foucaud *et al*, 2007; Hamilton Jr *et al*, 2008). The data highlight the importance of ROS in cell toxicity particularly in the presence of DPPC. DPPC appears

to increase oxidative stress within the cell in agreement with other studies where it was also found that its addition significantly increased ROS production in A549 cells (Herzog *et al*, 2009; Foucaud *et al*, 2007). An investigation into different variations of silica nanoparticles including iron- and titania- containing particles, noted increased levels of intracellular ROS generation. This was believed to occur through nanoparticle entry by a Trojan-horse type mechanism dependant on surface chemistry of particles (Limbach *et al*, 2007). A similar theory can be applied to this study and the increased oxidative stress observed following the addition of DPPC. It is possible that interaction with DPPC causes particles to have a higher level of dispersion, increasing the surface area available for interaction and therefore increasing the likelihood of cellular uptake - resulting in generation of ROS. It must also be considered that the surface chemistry and activity of particles is altered by DPPC, changing its toxicity, making the particles more reactive and thus more toxic (Herzog *et al*, 2009; Hamilton Jr *et al*, 2008; Buford *et al*, 2007).

The observation that cell death can occur via a number of pathways in A549 cells indicates a two tiered mechanism of cell death, cell cycle arrest and ROS mediated mitochondrial dependant cell death (Piao *et al*, 2011). The data in this study suggest that while not changing nanoparticle toxicity DPPC may influence death pathways as demonstrated by the induction of oxidative stress. The observation that DPPC leads to ROS generation in A549 cells suggests a possible switching from a ROS independent pathway as described by Chairuangkitti *et al*, 2013 causing cell cycle arrest to a ROS dependent pathway culminating in apoptotic cell death (Chairuangkitti *et al*, 2013). Further mechanistic studies are required to conclusively determine if pathway switching due to DPPC interaction is occurring.

6.5 Chapter Summary

This chapter has demonstrated the role of DPPC in modifying toxicity, namely inducing intracellular ROS production. Its interaction with AgNP highlights the importance of particle-surfactant interactions and the altered effects produced when in contact with mammalian cells. The data presented are in agreement with previous studies illustrating the impact on toxicity caused by nanoparticle contact with DPPC and the resulting alteration in ROS generation and inflammatory response. These findings may contribute

to developing a realistic risk assessment of nanoparticle exposure and the potential biological outcomes following entry and interaction with various fluids and surfactants present in the body.

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Chapter 7

Silver nanoparticle induce pro-inflammatory gene expression and inflammasome activation in human monocytes

7.1 Introduction

Silver nanoparticles (AgNP) are one of the most commonly utilized nanoparticles due to their antimicrobial properties (Duncan, 2011). As a result, the likelihood of exposure is becoming more frequent thus posing potential health risks that must be addressed. Cytotoxicity induced by AgNP exposure has been demonstrated in a number of studies with a variety of factors influencing observed cyto- and geno-toxic effects (Choi *et al*, 2010; Kim *et al*, 2009; Lankoff *et al*, 2012). Upon exposure AgNP have been shown to induce immune reactions at sub-lethal concentrations, including IL-8 induction and are involved in the generation of reactive oxygen species (ROS) (Lim *et al*, 2012). Following inhalation, nanoparticles have been shown to infiltrate the alveolar cell wall causing toxicity in alveolar macrophages which leads to dysfunction in the cytokine network by potentially penetrating the alveolar wall and infiltrating the bloodstream. This results in the translocation and distribution of nanoparticles to various organs and potential detection by circulating blood monocytes (Liu *et al*, 2013; Johnson *et al*, 2010; Kim & Choi, 2012).

An intact and functioning immune system is vital in the protection against harmful external stimuli. The controlled and rapid initiation and effective resolution of inflammation is vital to maintain normal homeostasis. Dysregulation of the immune response has been implicated in many human disease processes including autoimmunity, allergy and cancer (Lin & Karin, 2007; Scheller *et al*, 2011). The innate immune response is the first line of active defence and comprises a number of phagocytic cells with monocytes particularly, the primary cell type which are involved in the initial immune response by recognising distinct pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) via toll-like receptors (TLRs) on the cell surface (Matzinger, 1994; Beutler, 2009; Oberbarnscheidt *et al*, 2011). The subsequent release of cytokines from immune cells co-ordinate the immune response and are vital in determining the type of response required and can synchronize the various immunological elements required.

The production of cytokines must be tightly regulated in order to prevent an overstimulation of the immune response which may lead to the initiation of a pathological process. IL-1 β is one cytokine that is tightly regulated at the transcriptional and post-translational level by inflammasomes. Due to its vast influence both on innate immunity and the systemic response its production requires a two-step signalling process; an initial priming step resulting in gene expression followed by inflammasome activation which results in the cleavage of the inactive form essential for IL-1 β maturation and subsequent secretion (Dinarello, 2009; Schroder & Tschopp, 2010; Gross *et al*, 2011; Latz *et al*, 2013). Nanoparticles including titanium dioxide (TiO₂), silica and also AgNP have been shown to cause inflammasome activation resulting in IL-1 β release (Winter *et al*, 2011; Yang *et al*, 2012).

As the initial release of cytokines following exposure to an external threat is pivotal in mounting an effective immune response, the aims of this study were to investigate the ability of AgNP to induce such a response in a monocyte cell line and to compare the response to that which occurs from LPS stimulation alone. Following this investigation the immunological response of AgNP on primary human monocytes was investigated to determine if a similar response was induced following exposure. The AgNP induced transcription of pro-inflammatory cytokines- IL-1, IL-6 and TNF- α as well as well as release of IL-1 β as an indirect marker of inflammasome activation was investigated. This study aims to provide an insight into the activation of innate immune responses following AgNP exposure in human whole blood monocytes and provide an indication of the role of the inflammasome in this response by secretion of mature IL-1 β , which drives inflammation.

7.2 Results

7.2.1 Cytotoxicity Testing

The AB assay was performed to determine the active and cytotoxic concentrations of THP-1 cells to AgNP exposure so that IC₅₀ concentration values could be used to elicit a gene response for the follow up studies. A concentrationdependent reduction in cell viability was observed (figure 7.1) following AgNP exposure for 24 hours. IC₅₀ concentration was calculated as described previously. From this data it was determined

that the most active and cytotoxic concentrations of AgNP for the remaining gene THP-1 monocyte studies were 50 and 100 μ g/ml (IC₅₀).

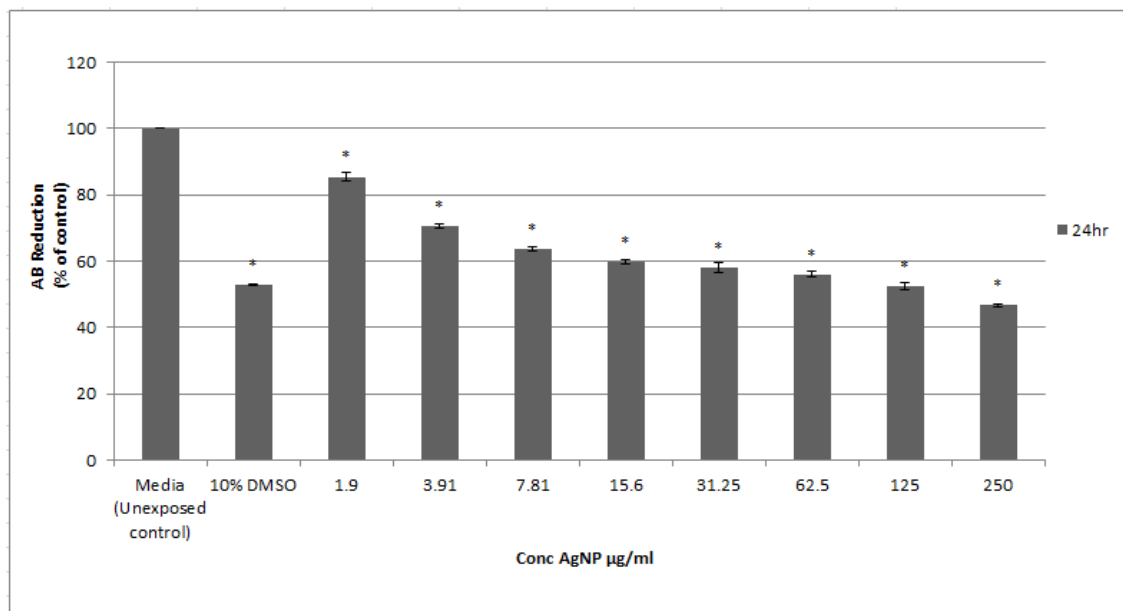


Figure 7.1 Cytotoxicity of AgNP in THP-1 cells after 24hr exposure as determined by the AB assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

7.2.2 THP-1 pro-inflammatory cytokine gene expression in response to AgNP exposure

The induction of pro-inflammatory gene expression by THP-1 monocyte cell line as a result of AgNP exposure was investigated. The response to AgNP at concentrations of 100 and 50 μ g/ml, based on IC₅₀ values, were tested. To determine immune response following exposure, transcript levels of pro-inflammatory cytokines IL-1, IL-6 and TNF- α in THP-1 monocytes were quantified by RT-PCR. Actin was used as the internal control. Results revealed a significant increase in transcript levels of all cytokines. In an experiment to establish possible endotoxin contamination of AgNP, THP-1 cells were incubated with polymyxin B (20 μ g/ml) for 1 hour prior to AgNP exposure. Endotoxin was not detected in AgNP used in this study (Appendix A7.2).

Induction of IL-1 transcript levels demonstrate a significant increase at both concentrations after 1, 6 and 8 hour exposures at 50µg/ml and after 6 and 8 hour exposures at 100µg/ml (figure 7.2) compared to the unexposed control. At all exposure concentrations a time dependent increase was noted peaking at 8 hours. At both concentrations, levels of IL-1 begin to decrease after 24 hour exposure. Levels of IL-1 at this time point were not significant compared to the unexposed control.

A statistically significant up-regulation in IL-6 induction (figure 7.3) was noted following all exposure time points at all exposure doses compared to the unexposed control. The increase in transcript levels occurs in a time dependent manner at AgNP concentration of 100µg/ml. IL-6 induction peaks 1 hour after exposure at a 50µg/ml concentration with a step-wise reduction observed with time. At 24 hours a significant increase in transcript levels compared to the unexposed control was noted at this concentration.

Induction of TNF- α (figure 7.4) was detected after 1, 8 and 24 hours at 50µg/ml with a significant increase in induction compared to the unexposed control. At the highest exposure concentration, 100µg/ml, up-regulation of TNF- α was detected after 1 and 6 hour exposures with a significant increase in transcript levels observed after 24 hours compared to the unexposed control.

The increase in transcript levels for TNF- α and IL-6 after 24 hours suggests prolonged induction and longer time points need to be investigated to determine if levels continue to rise.

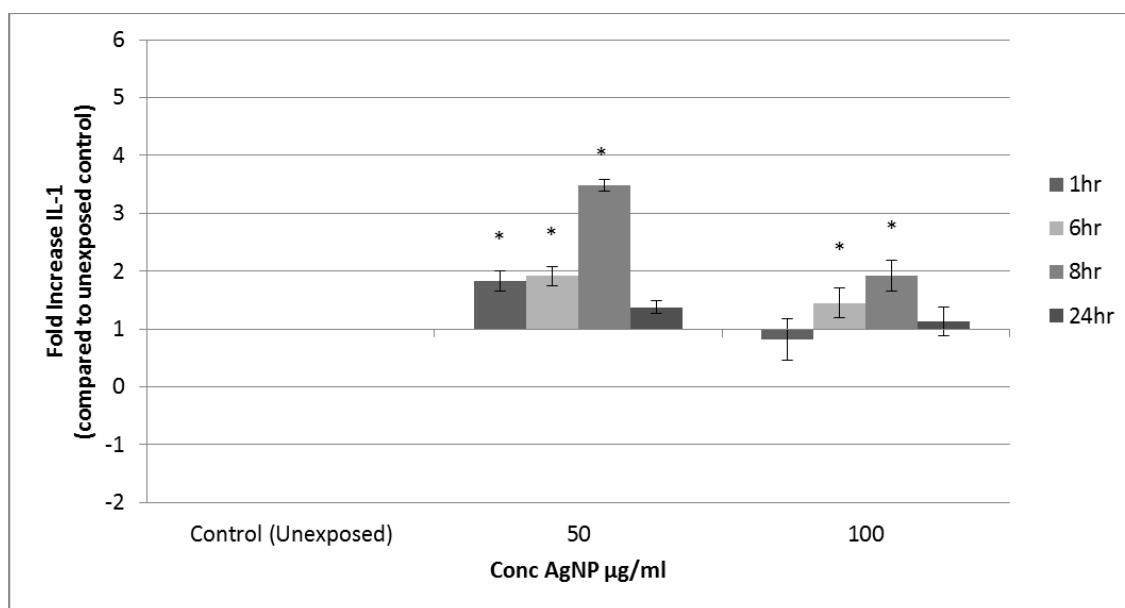


Figure 7.2 Fold increase in IL-1 induction in THP-1 cells exposed to 50µg/ml and 100µg/ml AgNP and compared to the unexposed control \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control. Actin was used as the internal control

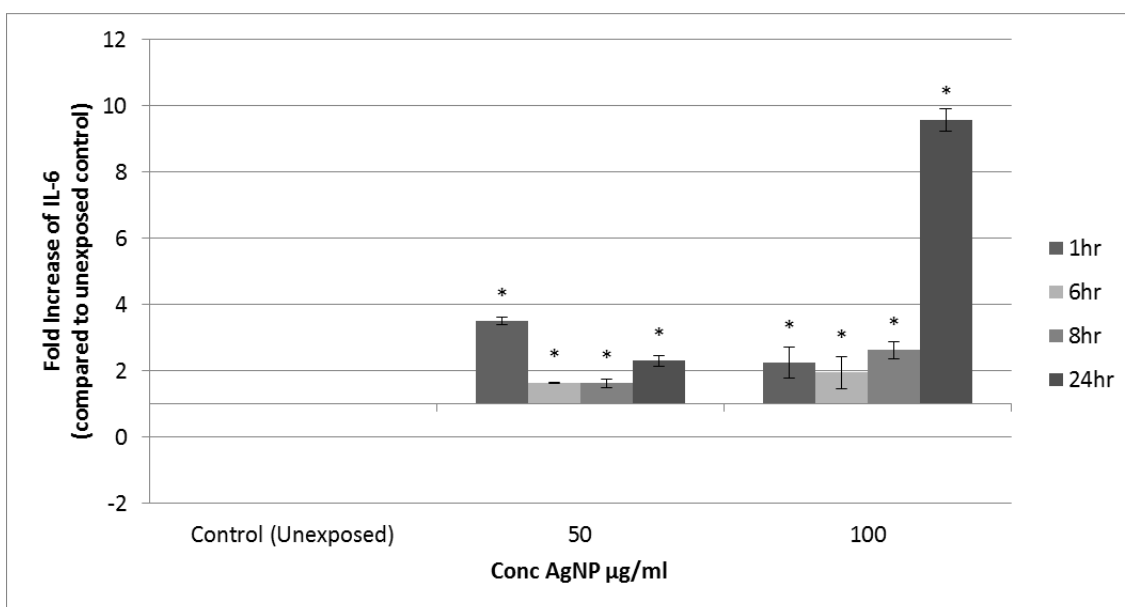


Figure 7.3 Fold increase in IL-6 induction in THP-1 cells exposed to 50µg/ml and 100µg/ml AgNP and compared to the unexposed control \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control. Actin was used as the internal control

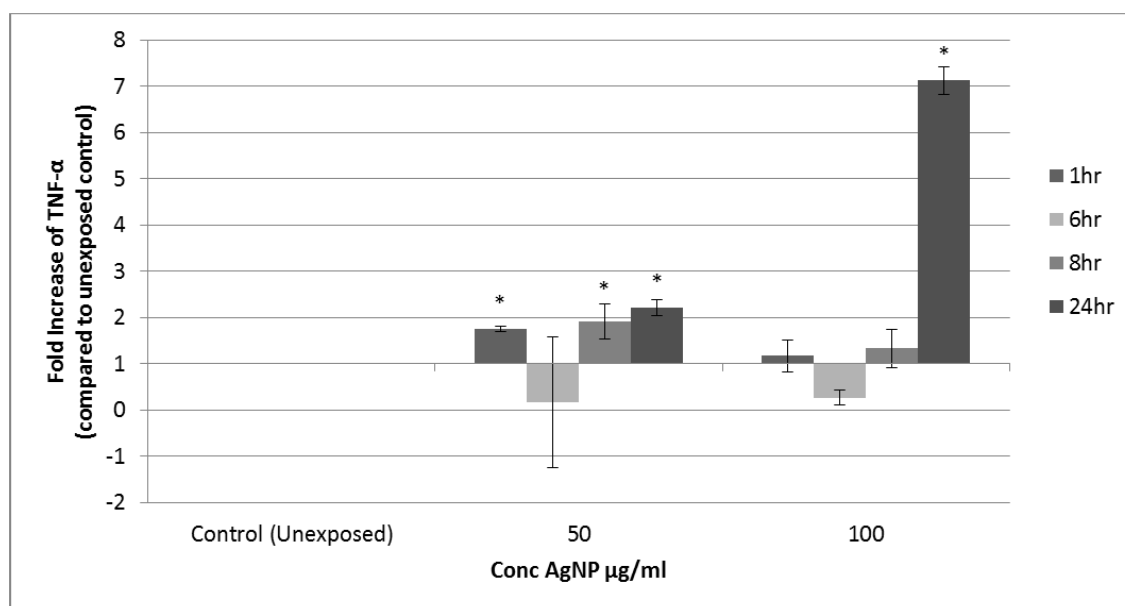


Figure 7.4 Fold increase in TNF- α induction in THP-1 cells exposed to 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ AgNP and compared to the unexposed control \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control. Actin was used as the internal control

7.2.3 Synergistic effect of LPS and AgNP on pro-inflammatory gene expression

AgNP were investigated for any potential synergism with LPS with respect to pro-inflammatory gene expression. Following exposure to a combination of LPS and AgNP, THP-1 cells demonstrated enhanced levels of gene expression compared to both the unexposed control and exposure to LPS or AgNP alone. Significant increases in IL-1 gene expression were observed at 6, 8 and 24 hours following LPS and combined 50 $\mu\text{g/ml}$ AgNP and LPS exposures (Figure 7.5). For exposure to combined 100 $\mu\text{g/ml}$ AgNP and LPS, significant increases compared to the unexposed control were noted at 6 and 8 hours. IL-6 induction (Figure 7.6) demonstrated significant increases in all exposure scenarios at 6, 8 and 24 hours compared to the unexposed control with significant increases in expression of TNF- α (Figure 7.7) observed at all time points and in all exposures compared to the unexposed control. For all of the cytokines, greater levels of induction were noted in the combined AgNP-LPS exposures compared to LPS exposure alone.

In addition to significant differences compared to the unexposed control student-newman-keuls multiple comparison tests were performed to determine if the difference in levels of expression between LPS exposed, AgNP exposed and AgNP-LPS exposed samples was significant. For IL-1 expression significant differences were noted for 6 (p<0.001), 8 (p<0.05) and 24 hours (p<0.01) between all exposure scenarios with the exception of 100µg/ml AgNP compared to 100µg/ml AgNP-LPS and compared to 50µg/ml AgNP-LPS which showed no significant difference in IL-1 induction after 24 hour exposure. Following 1 hour exposure no significant differences in IL-1 gene expression were observed. IL-6 expression demonstrated statistically significant differences between all exposure scenarios with p values of p<0.001 at all exposure time points. Levels of TNF-α up-regulation demonstrated a significant difference between all exposure scenarios at all time points, with p values of p<0.01 after 1 hour and p<0.001 after 6, 8 and 24 hours.

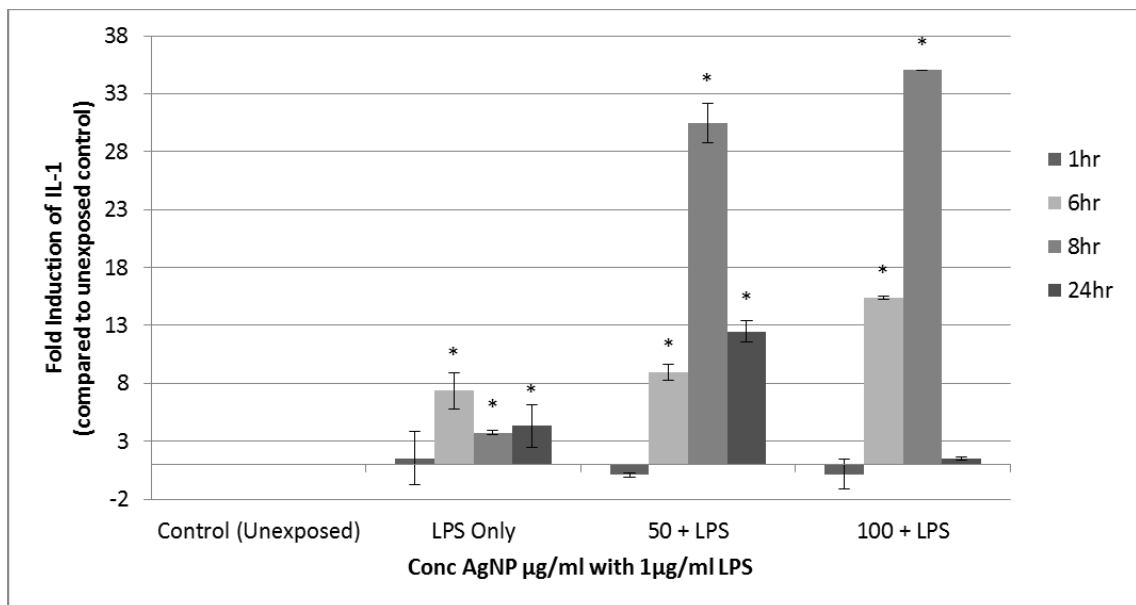


Figure 7.5 Fold increase in IL-1 induction in THP-1 cells following exposure to 1µg/ml LPS and a combination of AgNP and LPS compared to the unexposed control ± SD of three independent experiments. * denotes a statistically significant (p<0.05) difference from the unexposed control. Actin was used as the internal control

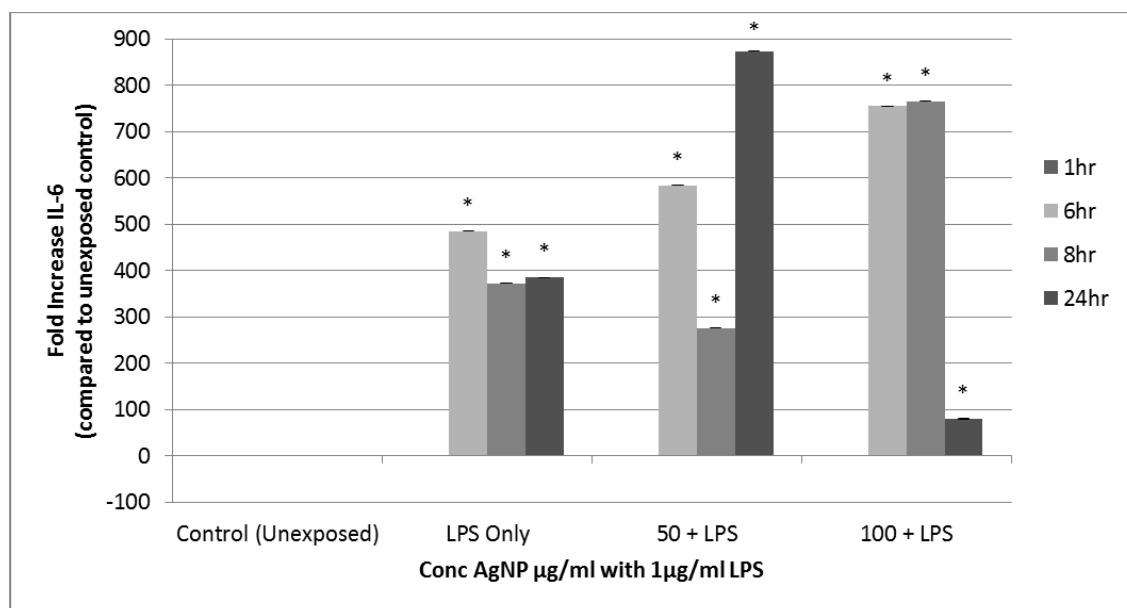


Figure 7.6 Fold increase in IL-6 induction in THP-1 cells following exposure to $1\mu\text{g/ml}$ LPS and a combination of AgNP and LPS compared to the unexposed control \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control. Actin was used as the internal control

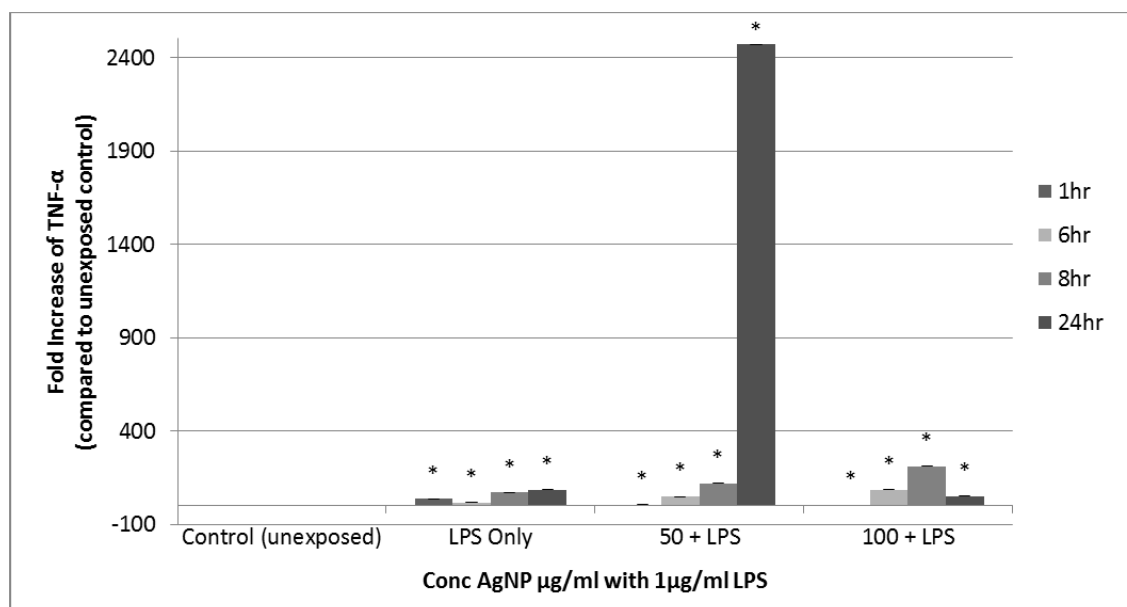


Figure 7.7 Fold increase in TNF- α induction in THP-1 cells following exposure to $1\mu\text{g/ml}$ LPS and a combination of AgNP and LPS compared to the unexposed control \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control. Actin was used as the internal control

7.2.4 Primary monocyte cytokine gene expression in response to AgNP exposure

Human blood monocytes were extracted from a cohort of 20 healthy donors and examined to determine whether a similar innate immune response was induced to the THP-1 monocytes. These monocytes were exposed only to a concentration of 50µg/ml AgNP for 8 hours due to the limited supply of monocytes extracted per donor sample. This exposure concentration and time point was chosen from the previous THP-1 studies as they appeared to be the most active test points.

The following results are presented for the average response to AgNP exposure in 20 healthy donors. As expected all donors displayed individual variation in response to AgNP for each cytokine. Therefore, the coefficient of variation (CV) was calculated for each cytokine between donors to determine the levels of variation between all blood donor samples. IL-1 demonstrated a CV of 63.7%, IL-6 54.5% and TNF- α 51.2%.

The results (figure 7.8) demonstrate general up-regulation of all cytokines in all donors responding to AgNP exposure compared to an unexposed control. Non-parametric statistical analysis was performed due to the donor samples being one-off samples. Combined levels (n=20) of IL-1 and TNF- α showed statistically significant up-regulation compared to the unexposed control as determined by the Mann-Whitney test. Figure 7.9 demonstrate the individual responses of 10 donors to AgNP exposure. As the data illustrate variation in individual gene expression was observed between donors as well as variation in the expression of each cytokine per donor. Kruskal-Wallis non-parametric analysis comparing the difference in expression levels of each individual cytokine between each of the 10 donors was found to be non-significant for all three cytokines. Spearman non-parametric assessment of differences between the levels of expression of the three cytokines per donor sample was shown to be non-significant.

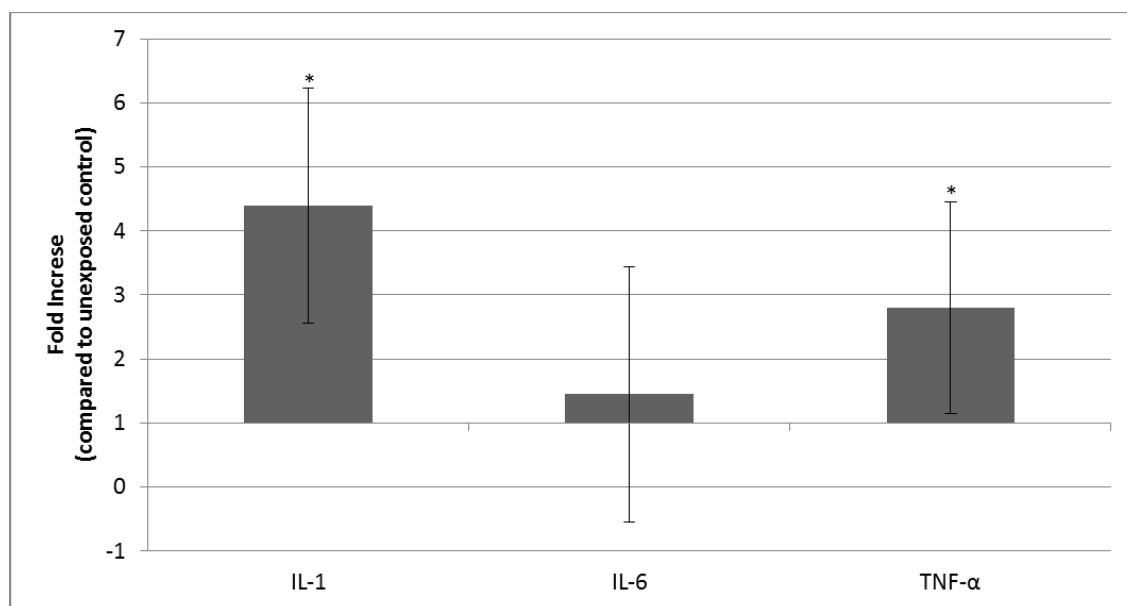


Figure 7.8 Fold increase in IL-1, IL-6 and TNF- α gene expression in primary monocytes following 8 hour AgNP exposure compared to an unexposed control \pm SD of twenty independent experiments. * denotes a statistically significant ($p < 0.05$) difference from the unexposed control. Actin was used as the internal control

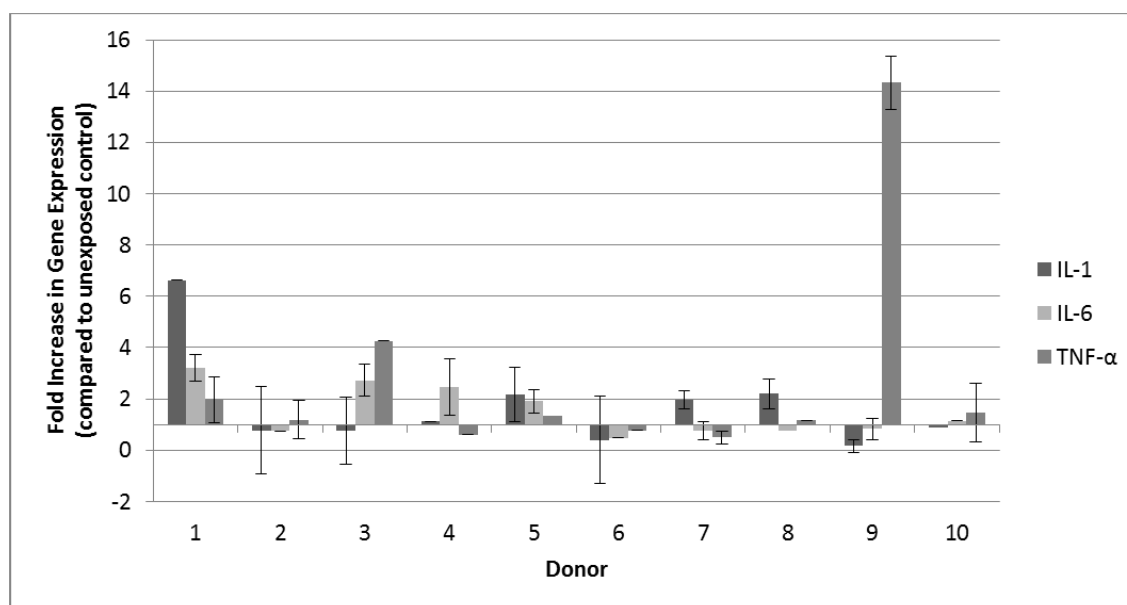


Figure 7.9 Fold increase in pro-inflammatory cytokine gene expression, IL-1, IL-6 and TNF- α in primary monocytes of 10 donors following 8 hour AgNP exposure compared to an unexposed control \pm SD of three independent experiments. Actin was used as the internal control

7.2.5 AgNP-induced inflammasome activation and cytokine secretion

Following the investigation into pro-inflammatory cytokine up-regulation by AgNP exposure, it was hypothesized that the induction of cytokine secretion could be due to inflammasome activation. The ability of AgNP to induce cytokine release following pre-treatment of cells with LPS was investigated to determine the potential inflammasome activation by AgNP themselves. An ELISA assay for IL-1 β secretion (Figure 7.10) was performed on the supernatant of THP-1 cells following 6 hour exposure to AgNP to detect inflammasome induced cleavage of pro-IL-1 β . The assay was also employed to confirm pro-inflammatory gene expression through detection of protein product, IL-1 β , in cell supernatant. Production of the protein confirmed inflammasome activation driven by AgNP exposure.

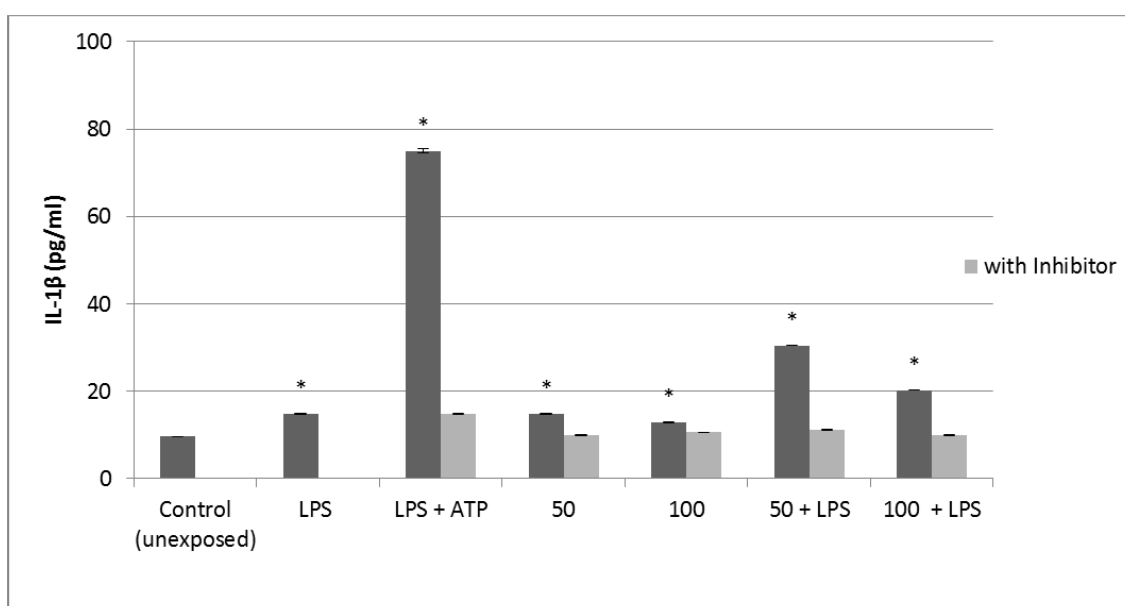


Figure 7.10 Release of IL-1 β (pg/ml) from THP-1 cells following 6 hour exposure to AgNP. LPS (10ng/ml) was pre-treated 4 hours before exposure and Ac-YVAD-CMK (20 μ M) inhibitor was treated 1 hour before exposure. ATP (5mM) was employed as a positive control. Data expressed as percentage of control mean \pm SD of five independent experiments. *denotes a statistically significant difference ($p < 0.05$) from the unexposed control.

An increase in IL-1 β levels compared to the unexposed control was noted for both exposure concentrations. While IL-1 β release was significantly increased compared to the unexposed control, levels were still low coming in under 20pg/ml. Following pre-treatment with LPS for 4 hours levels of IL-1 β release significantly increased to levels of 20pg/ml and above. The highest increase was noted at the 50 μ g/ml dose. Pre-treatment of cells with LPS only, resulted in a significant increase in IL-1 β release compared to the unexposed control similar to the levels observed for treatment of cells with AgNP only. The combined pre-treatment with LPS and exposure to AgNP caused significant IL- β release compared to the unexposed control and significantly higher levels when compared to AgNP and LPS exposure alone. This suggests a synergistic effect between AgNP and LPS in inflammasome activation.

Inclusion of the inhibitor prevented IL-1 β release in all exposure scenarios. The inhibitor is specific to caspase-1 which prevents the proteolytic cleavage of pro-IL-1 β into the mature active form. These results indicate a possible role of caspase-1 in AgNP mediated inflammasome activation.

7.3 Discussion

The prevalence of AgNP in consumer products as well as their use for the improvement of medical devices and in biomedical applications increases in the incidence of human exposure (Sotiriou & Pratsinis, 2011; Chen & Schluesner, 2008). Translocation and systemic distribution of nanoparticles has been documented and as such the influence of exposure in the circulatory system and the associated risk must be assessed (Johnson *et al*, 2010; Van der Zande *et al*, 2012; Loeschner *et al*, 2011).

In this study it was established that exposure to AgNP resulted in significant transcription of inflammatory cytokines IL-1, IL-6 and TNF- α . AgNP exposure can generate intracellular reactive oxygen species (ROS) in mammalian cells, with excessive production linked to cell damage and initiation of an inflammatory response (Lim *et al*, 2012; Asharani *et al*, 2009). Oxidative stress in monocytes coupled with the release of inflammatory cytokines is a natural protective response; however, this process can become pathogenic when normal cell control systems are overwhelmed. Evidence has indicated a correlation between ROS production and inflammation resulting in an amplified response. Oxidative stress causes expression of cytokines including IL-1, IL-6

and TNF- α which in turn contribute to ROS generation. This produces an amplification loop between oxidative stress and inflammation initiated by AgNP exposure (Kim & Ryu, 2013). This loop while potentially contributing to resolution may also cause continued pro-inflammatory cytokine induction past the threshold of resolution thus inducing a continued inflammatory response and toxicity (Lander, 1997; Acker, 2005; Franco *et al*, 2012). The pro-inflammatory cytokine expression detected following AgNP exposure and the amplified expression noted following exposure to AgNP and LPS combined may result from ROS production. These AgNP have previously been shown to be potent inducers of ROS (Murphy *et al*, 2015; Gupta Mukherjee *et al*, 2012). Further investigation is required to determine if a similar ROS driven mechanism occurs following exposure to these specific AgNP.

The increased gene expression observed by the combination of AgNP and LPS exposures indicated a synergistic effect and may further indicate a potential interaction which exacerbates the inflammatory response, potentially contributing to a greater immune reaction. As previously discussed, significant increases in levels of all cytokines compared to the unexposed control were noted. Significant differences in the expression of all cytokines after 6, 8 and 24 hours, were determined when comparing the differences in cytokine levels of cells exposed to either a combination of AgNP and LPS compared to AgNP, or LPS alone. An interaction between AgNP with LPS would explain the increased gene expression compared with exposure to AgNP or LPS alone. An important part of AgNP toxicity is attributed to nanoparticle-biomolecule interactions. Upon entry, nanoparticles associate with various biomolecules producing a surface coating. The variety of biomolecules within the body leads to any number of combinations coating the particle surface, making it an important contributory factor of cellular response to nanoparticles and the extent of toxicity (Murphy *et al*, 2015; Lynch *et al*, 2006; Monopoli *et al*, 2012; Dubey *et al*, 2015). A nanoparticles biomolecule surface coating can induce a Trojan horse effect whereby toxicity appears delayed only to occur days later, resulting from dissolution of the biomolecule coating within the lysosome following cellular internalisation (Wang *et al*, 2013). An interaction between AgNP and LPS must be considered, and it is clear that numerous factors contribute to the responses induced. Further investigation is required to determine this interaction with particular attention paid to the biomolecule surface coating in order to understand the complete effect of AgNP exposure.

Following investigation into gene expression by AgNP exposure in THP-1 cells, human blood monocytes extracted from a cohort of 20 healthy donors were exposed to AgNP to determine if a similar innate immune response was induced. While significant up-regulation in IL-1 and TNF- α compared to the unexposed control was observed when comparing the donors as a whole, variation between individual donors in their expression of a particular cytokine and variation between the levels of the three cytokines expressed by each donor was also noted. Even under the same experimental conditions variation in gene expression was expected. There is evidence of donor-specific gene expression patterns which can be attributed to the data shown here and also the large error observed when the expression patterns for a particular cytokine were grouped and averaged. Statistical analysis of the variation, which ranged from 51.2-63.7%, were performed but indicated the variations between donors were non-significant. Several studies involving donor blood samples have demonstrated variation in gene expression patterns with each donor producing differing levels following exposure to a particular stimulus. Many factors can contribute to the variation in expression, from environmental and physiological factors to the time of day of sample taking (Howe *et al*, 2005, 2009; Whitney *et al*, 2003). The inflammatory response in human circulating blood is a complex systemic process involving numerous factors which contribute to the large CVs and varied results. A larger cohort of samples to include other cells per donor such as lymphocytes would be an interesting investigation to elucidate this response on a more clinical level.

The effect of AgNP on the release of IL-1 β and its potential to cause inflammasome activation yielded some interesting results. Exposure to AgNP alone resulted in a low but significant level of IL-1 β release compared to the unexposed control. Pre-treatment of cells with LPS followed by AgNP resulted in significantly higher IL-1 β release indicative of a synergistic relationship between the two. This result corresponds to the increase in gene expression observed following combined AgNP-LPS exposure. This effect on IL-1 β release may potentially have pathological consequences and result in a greater immune response produced by minimally activated cells when AgNP are present. Given their commonality in consumer products and the incidence of exposure, their presence may trigger an exacerbated immune reaction in what would have been a minimal immune response. Another interesting observation was that the lower concentration exposure produced the greatest release indicating that at sub-lethal

concentrations of AgNP a greater response is induced, and this is an important dose-metric consideration. This type of reaction has been observed in other studies examining AgNP exposure which the findings here support (Lim *et al*, 2012; Yang *et al*, 2012). The inclusion of an inhibitor highlights the involvement of caspase-1 in inflammasome activation and potentially the specific involvement of the NLRP3 inflammasome which has been shown to become activated upon AgNP exposure (Yang *et al*, 2012; Simard *et al*, 2015). The involvement of caspase-1 would explain the low levels of IL-1 β release following cell exposure to AgNP or LPS alone as it has been shown that monocytes constitutively express activated caspase-1 and as a result only require one stimulating event in order to cleave pro-IL-1 β , which may explain the low levels detected following exposure to AgNP or LPS alone (Netea *et al*, 2009).

The first signal in inflammasome activation leading to production of pro-IL-1 β is often NF- κ B. AgNP have been shown to stimulate this pathway resulting in downstream effects ranging from cell cycle arrest to apoptosis (Eom & Choi, 2010). A causative mechanism of NF- κ B expression is ROS, which can lead to several inflammatory responses including mitochondrial DNA release, causing NLRP3 inflammasome activation (Gloire *et al*, 2006; Shimada *et al*, 2012; Hehner *et al*, 2000). AgNP entry into cells is believed to occur via receptor-mediated endocytosis, and evidence suggests that AgNP can activate toll like receptor pathways, another mechanism of NF- κ B activation (Kim *et al*, 2007; Kim *et al*, 2012). It is possible that TLR-2 is involved as it has been shown that AgNP induced apoptosis can be partially mediated by the TLR-2 signaling pathway (Kim *et al*, 2012; Dubey *et al*, 2015). Interaction of AgNP with a TLR coupled with AgNP induction of NF- κ B highlights a possible mechanism for inflammasome activation. Further investigation into specific TLR-AgNP interactions is required to determine any involvement. The heightened gene expression to combined AgNP-LPS exposure may suggest involvement of the TLR-4 signaling pathway, a receptor that specifically recognizes LPS, as another potential initiation event in inflammasome activation.

ROS, specifically mitochondrial superoxide, have been implicated in the formation of the inflammasome complex. Two additional mechanisms have also been identified, one involves interaction of AgNP with cell membrane affecting integrity and resulting in alteration to K⁺ efflux channels, the other involves cathepsin release following particle entry into the lysosome which in turn affects mitochondrial function resulting in ROS

release and thus inflammasome formation (Yang *et al*, 2012). Another contributor to inflammasome activation by AgNP was recently identified as degradation of a stress sensor associated with the endoplasmic reticulum brought on by exposure (Simard *et al*, 2015). Further investigation is required to determine if one or all of these mechanisms are responsible for inflammasome activation by the specific AgNP utilized in this study. In addition, AgNP have been investigated for their potential adjuvant properties with studies indicating a significant adjuvant effect in mice (Xu *et al*, 2013). This evidence of the immunostimulatory effect of AgNP highlights the immunological significance of AgNP and is in agreement with the data presented in this study regarding gene expression and IL-1 β release induced by AgNP exposure alone and the increased levels released following the combined exposure to AgNP and LPS.

7.4 Chapter Summary

This chapter has highlighted the immunological significance of AgNP exposure and consideration must be given when assessing the influence of AgNP on the cells of the immune system. These results indicate that AgNP could potentially have pathological implications or indeed exacerbate already existing pathological conditions.

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Chapter 8

General Discussion and Future Work

8.1 Discussion

This thesis has clearly demonstrated that AgNP exposure can induce an innate immune response in monocytes through pro-inflammatory cytokine up-regulation with inflammasome involvement. In addition this study has illustrated the importance of components of biological fluids in the modification of AgNP toxicity. In chapter 1 a number of questions were asked regarding AgNP toxicity and their influence on biological systems following oral exposure, below the main findings of this thesis are identified and discussed.

To date the work performed for this project has included:

1. Characterisation of pristine AgNP and AgNP in the presence of biofluid components UDCA, CA, DCA and DPPC, identifying any modifying effects
2. Cytotoxic evaluation of AgNP on multiple cell lines associated with oral exposure, liver, larynx and lung (HepG-2, Hep2 and A549 respectively)
3. Investigation into the modifying effects of the aforementioned biofluid components on the observed toxicity
4. Investigation into the innate immune response induced by AgNP exposure on a monocyte cell line (THP-1)
5. Investigation into the innate immune response induced by AgNP exposure on primary human monocytes and how this compares to the response induced in THP-1 cells
6. Identifying the involvement of the inflammasome complex in AgNP induced innate immune response

The aim of this work was to fully characterise AgNP and to explore their toxic potential on a variety of cell lines associated with oral exposure. Following this, the ability of certain biofluid components associated with oral exposure to modify the cytotoxicity of AgNP was established. Prior to cytotoxic evaluation, it was determined if the presence of biofluid components could alter the physicochemical characteristics of AgNP. Most

cyto-toxicological studies are aimed at the risk associated with dermal exposure and inhalation, this study aimed to assess the risk of oral exposure to AgNP to include ingestion but also inhalation, as the two systems operate millimetres apart and the likelihood of cross exposure between the two is unavoidable. Following initial investigation, this study went on to focus on the potential innate immune response induced by AgNP in circulating white blood cells following absorption into the blood stream. The focus of this work was to mimic more realistic exposure scenarios and concentration metrics associated with real world nanoparticle exposure. The interaction between humans, the environment and nanoparticles is becoming more widespread and frequent, highlighting the importance of establishing the real risks associated with exposure.

The first step in any cyto-toxicological investigation is to establish the physicochemical characteristics of the nanoparticle of interest. A number of methods including scanning electron microscopy, dynamic light scattering analysis and UV/VIS absorption spectroscopy were performed independently to provide a complete picture of the characteristics of AgNP. BET was also employed to determine the surface area of particles. All techniques were performed on the particle powder as purchased. Analysis revealed a particle size between 50-70nm by SEM. DLS analysis which determines the hydrodynamic radius of particles, revealed an average size of $34\text{nm} \pm \text{SD } 3.5$. BET analysis on the nanoparticle powder revealed a surface area of $2.3 \pm 0.1 \text{ m}^2/\text{g}$. The same techniques were also performed on the AgNP in the presence of the biofluid components under investigation, UDCA, CA, DCA and DPPC to determine any alteration to the size or agglomerative state of the particles. On the addition of biofluid components to nanoparticle suspensions dispersed in the appropriate cell culture media (RPMI or DMEM); DLS revealed a shift in particle size in the presence of DPPC and UDCA but remained within the size range observed for AgNP dispersed in media alone. Following addition of UDCA, CA and DCA (dispersed in DMEM); particle size was revealed to fall within the size range for the cell culture media. It has been observed that upon entry into the body a material becomes almost immediately coated in protein and this may be the case upon addition of the various biofluid components. These components may associate with and coat the particle upon contact causing alterations to particle size. However it has also been demonstrated that proteins contained in cell culture media can also associate with particles and it can be assumed that this is also

occurring. With this in mind it would be expected that the association or coating with various proteins would cause particles to agglomerate and an increase in particle size observed but, neither scenario occurs. The lack of an increase in particle size in the case of UDCA and DPPC (dispersed in RPMI) may be attributed to a shift in the plane of shear. This shift was also noted when particles were dispersed in cell culture media only. Addition of UDCA, CA or DCA resulted in a large shift in hydrodynamic radius demonstrating one peak falling within the size range of DMEM. These biofluid components have a large size distribution (greater than 200nm) and may cause the formation of large agglomerates with AgNP, resulting in precipitation of particles out of solution at an elevated rate preventing their detection by DLS analysis. This may explain why only one peak is observed following analysis of these dispersions which is attributed to DMEM. Further investigation is required to determine why these particular AgNP-biofluid dispersions cannot be detected by DLS analysis. Whatever the process may be interaction between AgNP and biofluid components may potentially influence their interaction with living systems, their distribution and the biological responses they induce.

In terms of producing a toxic response in cells, it was important to establish whether AgNP could interact with the components and various growth factors of cell culture media thus influencing toxicity. Any interaction with media components could lead to a secondary toxicity due to depletion of vital growth factors essential for cell survival. UV/VIS absorption was employed to study this effect. The spectra revealed all of the features for cell culture media at 360, 410 and 560nm with AgNP and DPPC, UDCA, CA and DCA revealing no features. The results indicated no interaction of particles with cell culture media or biofluid components. It was concluded that no secondary toxicity occurs due to depletion of media components.

Zeta potential analysis was also carried out to assess the overall stability of AgNP in the various dispersions. Values varied among the suspensions. The overall system is stable at values above +30mV and below -30mV. Analysis of AgNP dispersed in dH₂O was unstable at all test concentrations. Dispersion in RPMI was shown to stabilize the system and with the addition of DPPC showed no change. Similar stability data was obtained for AgNP dispersed in DMEM and addition of UDCA, CA or DCA had no effect. The lack of change in zeta potential values corresponds to the DLS data where no

significant change in hydrodynamic radius was observed. A change in hydrodynamic radius, indicating interaction between particles and biofluid components, would correspond to a change in zeta potential values suggesting indirect toxicity resulting from an interaction (Casey *et al*, 2007; Casey *et al*, 2008). The large values noted for AgNP in RPMI with DPPC and UDCA correspond to the DLS data, suggesting that particles have a tendency to repel each other preventing the likelihood of agglomeration and a change in size. The low unstable values noted for AgNP dispersion in DMEM with UDCA, CA and DCA while indicating no change in the overall system may explain the lack of particle size data. The lower values suggest a reduced repulsion force increasing the tendency to agglomerate (Xia *et al*, 2006). The addition of these biofluid components together with DMEM may result in agglomeration and thus precipitation of particles out of solution preventing detection by DLS analysis. However no significant changes were noted indicating biofluid components do not affect the overall stability of cell media-nanoparticle system.

Following physicochemical characterisation of AgNP and establishing their interactions with the selected biofluid components, the ability of these components to modify toxicity was assessed. A number of toxicological tests were performed to determine the toxic effect induced by AgNP on two cell lines associated with the GI tract, liver (HepG-2) and laryngeal (Hep2) and if biofluid components associated with these tissues had modifying effects. In terms of the effect of UDCA on the HepG-2 cell line, no change in toxicity was noted however, CA and DCA did have modifying effects. The pattern of toxicity as demonstrated in the AB and MTT viability assays remained unchanged following addition of CA and DCA to the system but significant changes were observed in the levels of ROS generated. The levels of ROS were significantly greater in AgNP-CA and AgNP-DCA dispersions compared to AgNP exposure alone. The increased production of ROS is potentially due to increased levels of lipid peroxidation caused by the combination of AgNP and biofluid components. Lipid peroxidation is a naturally occurring process driven by ROS but in an uncontrolled state may lead to altered cell structure and function (Mylonas & Kouretas, 1999). It has been demonstrated that certain nanomaterials such as ZnO nanoparticles can enhance this process causing increased ROS production with AgNP also implicated in this process due to the quenching of natural antioxidant systems and increased ROS generation (Premanathan *et al*, 2011; Adeyemi & Faniyan, 2014). From the data it is postulated

that it is this mechanism of cell destruction occurring as the levels of ROS are substantially increased in the presence of both AgNP-CA and AgNP-DCA compared to AgNP alone. While exposure to AgNP alone can result in an increase in lipid peroxidation it is possible that the addition of CA and DCA can result in an escalation of this process which then has a knock on effect on cell viability. This may be the result of a reactive product produced from the interaction of AgNP with CA and DCA. Further investigation is required to determine the mechanism of cell death and the role of lipid peroxidation in the pathway.

As a continuation of this investigation into biofluid modification of toxicity, a laryngeal (Hep2) cell line was investigated to establish if the biofluid components could have modifying effects on areas of the body outside their normal target organs. For this UDCA was chosen as the biofluid component to be investigated. A significant modification in AgNP toxicity was observed in this study. A delay in cytotoxicity was noted at 24 hour exposure with a significant reduction in viability following prolonged exposure (48, 72 and 96 hours). This delay was coupled to a significant reduction in ROS generation following UDCA addition suggesting a possible antioxidant effect of UDCA within the first 24 hours after exposure. It is possible that UDCA causes a delayed toxicity following association with AgNP in the first 24 hours due to a Trojan horse type mechanism after which particle trafficking and dissolution within the cell results in degradation of UDCA leaving the “naked” AgNP free to cause toxicity (Wang *et al*, 2013; Mwilu *et al*, 2013).

The biofluid components investigated in this thesis are employed as pharmaceutical agents and medications and as such highlights their potential to be present systemically and not just confined to the liver. As a result the observations in this thesis are a relevant line of investigation particularly as these bile acid derived treatments are given orally. The data highlight the need for a complete and detailed cytotoxic profile of AgNP and not just of the isolated effects but of the combined effect encompassing the local environment, cellular components and other contributors at the exposure site.

As exposure through inhalation is the most common route of nanoparticle entry a follow up study was undertaken to investigate the interaction of a component of lung surfactant, DPPC, with AgNP and its potential to influence toxicity and induce an

inflammatory response. Toxicity data demonstrated the effect of AgNP on the viability of a lung cell line, A549. Due to the rapid doubling time of this cell line, 22 hours, the greatest level of toxicity was at higher doses and shorter exposure times (24-48 hours). The apparent cell recovery at lower AgNP concentrations at 72 and 96 hours was attributed to replication of the surviving cells. Long term viability studies demonstrated a reduced capacity for cell proliferation and colony formation in the presence of DPPC. The greatest modification in toxicity was noted in the generation of ROS in the presence of DPPC compared to substantially lower levels generated following exposure to AgNP alone. The induction of oxidative stress plays a central role in cell death caused by AgNP exposure. AgNP are potent inducers of oxidative stress and coupled with their ability to diminish natural antioxidant stocks are critical in initiating apoptosis (Martindale & Holbrook, 2002; Sastre *et al*, 2000; Piao *et al*, 2011; Arora *et al*, 2008; Hussain *et al*, 2005; Chairuangkitti *et al*, 2013). Lipid peroxidation may also play a role in this process enhancing ROS generation (Premanathan *et al*, 2011; Adeyemi & Faniyan, 2014). This cell death process requires involvement of the mitochondria however another death pathway has been identified in this particular cell line which does not require the induction of ROS. This ROS-independent pathway results in cell cycle arrest halting proliferation rather than inducing apoptosis (Chairuangkitti *et al*, 2013). It is possible that the results observed in this thesis indicate death pathway switching following addition of DPPC. Exposure to AgNP alone produced low levels of ROS at high concentrations only but addition of DPPC resulted in significant ROS induction at a number of different concentrations suggesting a switch from a ROS-independent mechanism causing cell cycle arrest to a ROS-dependent mechanism culminating in apoptotic cell death.

The induction of oxidative stress resulting from AgNP exposure has a number of important knock on effects including cellular and DNA damage and the induction of inflammatory cytokine release (Nel *et al*, 2006). In this study the release of IL-8 and TNF- α were investigated and these particular cytokines have been associated with AgNP exposure and implicated in certain lung disorders (Singh *et al*, 2007; Lim *et al*, 2012; Park *et al*, 2011; Napierska *et al*, 2012). While AgNP exposure resulted in their release from A549 cells, the addition of DPPC caused a significant increase in release. This implicates AgNP as an initiator of inflammation with its interaction with DPPC serving to increase its potency. The increased levels and immediate release of IL-8 in

particular support reports of its potential use as a bio-marker of acute inflammation in response to AgNP exposure (Lim *et al*, 2012; Park *et al*, 2011). The alteration in both IL-8 and TNF- α release caused by DPPC suggest the potential to induce a chronic inflammatory state due to high levels released at prolonged exposure times. The data also suggest that it is at sub-toxic doses of AgNP that produce the greatest inflammatory response which has previously been reported (Lim *et al*, 2012). The alterations in ROS generation and inflammatory cytokine release by DPPC can possibly be explained by interactions between this surfactant and AgNP. Any potential interaction may increase particle dispersion or alter the surface chemistry resulting in greater surface area available for interaction increasing the likelihood of cellular uptake and cellular trafficking. A similar Trojan horse mechanism has been suggested for other nanoparticles which result in increased levels of ROS generation (Limbach *et al*, 2007). It is possible that DPPC-AgNP interaction results in alteration to particle characteristics producing a more reactive and thus more toxic particle. The results indicate that not only are sub-lethal concentrations of AgNP potent inducers of an inflammatory response but that the favoured protective surface coating DPPC is a major contributor to oxidative stress induction.

The final part of this thesis involved identifying the ability of AgNP to induce an innate immune response in circulating blood cells. Due to the possibility of AgNP translocation into the blood stream it is an important part of any risk assessment to investigate any potential effects on the circulatory system (Loeschner *et al*, 2011; Gaillet & Rouanet, 2015). AgNP exposure to THP-1 cells showed a significant up-regulation in gene expression of IL-1, IL-6 and TNF- α . Oxidative stress has been implicated in the up-regulation of inflammatory cytokines. The induction of pro-inflammatory cytokines by ROS generation can result in a positive feedback loop whereby the subsequent cytokine expression further drives the production of ROS (Kim & Ryu, 2013; Lander, 1997; Acker, 2005; Franco *et al*, 2012). While this mechanism may result in resolution, uncontrolled amplification may cause this process to turn pathogenic, eventually resulting in toxicity. The cytokine up-regulation following AgNP and combined AgNP-LPS exposure may result from ROS production. As these AgNP have previously been shown to be potent inducers of ROS in certain mammalian cell lines, further investigation is required to determine if this mechanism is what drives the innate immune response caused by exposure (Gupta Mukherjee *et al*, 2012; Lim *et al*,

2012; Asharani *et al*, 2009). The increased gene expression following combined exposure to AgNP and LPS indicated a synergistic relationship between the two. This combined effect produced pro-inflammatory cytokine gene expression at significantly higher levels compared with exposure to AgNP or LPS alone. An interaction between AgNP and LPS would explain the significantly increased levels generated. An important consideration in nanoparticle toxicity is the interaction with various biomolecules following exposure. Nanoparticle interaction with biomolecules can result in particle coating and this coating has the potential to determine the biological responses induced and also determine particle trafficking through the body (Lynch *et al*, 2006; Laurent *et al*, 2012; Monopoli *et al*, 2012; Dubey *et al*, 2015). It must be considered therefore the potential interaction between AgNP and LPS, in particular the biomolecule surface coating, and how this contributes to the augmented response.

As a continuation of the initial investigation into AgNP induced immune response, an identical set of experiments was performed on primary human monocytes extracted from a cohort of twenty healthy donors to determine if a similar response was generated in primary cells. The results demonstrated significant up-regulation of IL-1 and TNF- α compared to the unexposed control. The main finding from this study was the large variation between donors in the levels of expression and also the variation in the expression of each cytokine by each donor. These results while demonstrating large errors only highlight the importance of donor variation and agree with existing evidence of donor specific responses and individual variation. Studies involving blood samples have noted donor-specific gene expression patterns with different levels of a particular target generated in response to the same stimulus and under identical conditions. The reasons for this varied response can range from environmental factors to the time of day of sample taking (Howe *et al*, 2005, 2009; Whitney *et al*, 2003). Although AgNP were shown to induce pro-inflammatory cytokine gene expression in primary monocytes, the variation between donors must be considered and further investigations into AgNP immune responses should be performed using a larger cohort of samples and perhaps also the inclusion of other donor cells to form a complete account of the response.

Following the determination of pro-inflammatory cytokine up-regulation, an investigation into the release of IL-1 β protein was performed to determine if inflammasome activation plays a role in AgNP mediated immune response. Low levels of IL-1 β were released following AgNP exposure but pre-treatment with LPS followed

by AgNP exposure produced the greatest levels of release. As with the gene expression data this may suggest an interaction between the two. This result may have pathological consequences potentially producing a greater immune response in minimally active cells due to the presence of AgNP. The results also demonstrated that at sub-lethal concentrations of AgNP the greatest IL-1 β release was observed which has been detailed in other investigations into the inflammatory activity of AgNP (Lim *et al*, 2012; Yang *et al*, 2012). The reduction in IL-1 β release following the inclusion of an inhibitor suggests the involvement of caspase-1 and specifically the activation of the NLRP3 inflammasome upon exposure. Its involvement may also explain the low levels of IL-1 β release following exposure to AgNP or LPS alone as monocytes have been shown to constitutively express caspase-1 allowing cleavage of pro-IL-1 β into the bioactive form and its subsequent release from the cell requiring (Yang *et al*, 2012; Simard *et al*, 2015; Netea *et al*, 2009).

Inflammasome activation involves a two signal sequence. The first stimulates gene expression; the second involves cleavage of the inactive cytokine to the mature active form. The first signal is often NF- κ B and AgNP have been shown to stimulate this pathway resulting in downstream effects ranging from cell cycle arrest to apoptosis. ROS have been shown to cause NF- κ B induction with AgNP contributing to ROS generation. AgNP have also been shown to interact with TLRs which can then lead to activation of the NF- κ B signalling pathways. TLR-2 in particular has been involved in AgNP mediated apoptosis and due to the interaction of AgNP with LPS it is also a possibility that TLR-4 may be involved as it specifically recognises LPS (Kim *et al*, 2007; Kim *et al*, 2012; Dubey *et al*, 2015). The interaction of TLRs with AgNP highlights a possible initiation event in inflammasome activation involving NF- κ B. Additional mechanisms have also been identified in AgNP mediated inflammasome activation. ROS, specifically mitochondrial superoxide, have been implicated together with alteration in K⁺ efflux channels and cathepsin release causing mitochondrial dysfunction and ROS generation, all resulting from AgNP exposure (Yang *et al*, 2012). Another contributor to inflammasome activation by AgNP was recently identified as degradation of an endoplasmic reticulum stress sensor (Simard *et al*, 2015). Further investigation is required to determine if one or all of these mechanisms is responsible for inflammasome activation and subsequent IL-1 β release brought on by exposure to the specific AgNP investigated in this study.

The antimicrobial potential of AgNP has led to their application in a variety of areas ranging from medical devices and drug therapies to cosmetics. The continuing growth of this industry means that exposure is becoming more common, which has highlighted a lack of information regarding the toxic potential of AgNP and other nanomaterials. The results of this study could provide preliminary information relating to the toxic potential of AgNP and more importantly provide a more realistic picture of their interaction with biological systems, and how this can influence nanoparticle interaction with living systems upon exposure. The results presented here also lend themselves to providing a preliminary insight into the toxic effect of AgNP upon oral exposure, the modifying effects on toxicity upon interaction with biofluids and the immune response provoked following absorption into the bloodstream, and finally, highlighting that the application of nanomaterials into consumer products should be approached with caution.

This report has highlighted that a certain level of caution should be undertaken when considering incorporating nanomaterials into consumer products. It is of the upmost importance that a certain level of care be taken when considering nanomaterials for consumer use, especially following documentation of the harm caused by these materials at low doses. When trying to establish the risk of such materials, it is important to take into account realistic parameters such as concentration metrics and interaction of nanomaterials with the individual components within living systems when designing experiments. This will provide accurate information on the potential hazards of nanomaterials towards humans and the environment. This information along with the results presented in this study will impact a number of sectors across the area of nanotechnology including, food and agricultural, food packaging, medical and cosmetic.

8.2 Future Work

8.2.1 Effect of AgNP on adaptive immunity

Furthering the investigation into the innate immune response generated in monocytes by AgNP exposure, an experiment designed to determine AgNP effects on the adaptive immune response will be investigated. Specifically this experiment seeks to identify the allosteric T cell response induced by AgNP exposure. This study would determine the ability of AgNP to induce T cell proliferation, important cells in the immune response

involved in destruction of infected cells and maturation of other immune cells. Following whole blood collection, both monocytes and lymphocytes will be extracted, specifically CD3 cells, from donor blood. Monocytes will be pre-treated with AgNP as in previous experiments and lymphocytes will be fluorescently labelled. Monocytes will then be combined with lymphocytes from another donor in a 96 well plate with cell media containing no cytokines and incubated. Following 3, 4, 5 and 6 days cells will be stained for CD3 (a marker present on both T helper and T killer cells) CD4 and CD8, and analysed on a flow cytometer. A histogram illustrating several peaks indicates cell division and would suggest that AgNP exposure can activate monocytes, which leads to the activation of T lymphocytes and subsequent cell proliferation, indicative of an adaptive immune response. The staining of specific CD markers would also identify the type of T cells induced by AgNP exposure.

8.2.2 Innate immune response of AgNP in an in vivo model

An interesting and important continuation of this work would be to determine if the response observed in both the monocyte cell line and in primary human monocytes is similar to the response induced in an in vivo model. *Galleria mellonella* larvae, a type of moth, will be used as an in vivo model to determine if a similar innate immune response to AgNP exposure is induced. *G. mellonella* are widely used in research for in vivo investigations into immune responses as the innate immune response is similar to the human innate immune response. The larvae will be injected with AgNP at a concentration of 50µg/ml and the hemolymph extracted for cellular extraction, RNA isolation and subsequent molecular analysis. The same endpoints IL-1, IL-6 and TNF-α will be analysed to determine cytokine up-regulation induced by AgNP exposure. These larvae are a useful in vivo model and can provide an important insight into the mammalian innate immune response following AgNP exposure.

8.2.3 Investigation into the toxicity of other metallic nanoparticles following oral exposure

This thesis has demonstrated important results regarding the effects of AgNP on biological systems following oral exposure. While there are still further investigations remaining to form a complete cytotoxic profile of AgNP exposure, it is important that

other nanoparticles are considered for similar investigation. Metallic nanoparticles such as titanium dioxide (TiO₂) are also applicable in consumer goods making the risk of exposure to the public more prevalent. TiO₂ is used in powdered foods such as powdered milk to prevent caking and is also used as a whitening agent in certain foodstuffs (Lomar *et al*, 2002; Smolkova *et al*, 2015). TiO₂ provides a good example of a metallic nanoparticle that should be considered for investigation as they are consistently used in consumer products especially food products making the risk of exposure a certainty. As such a complete cytotoxic profile of these nanoparticles must be performed to determine the potential risks posed following exposure. Similar to the work undertaken in this thesis, investigations into the possible interaction with various biological entities and their ability to induce a biological response must be considered. This work will add to the knowledge available regarding potential hazards of nanoparticle use in consumer products, not as a barrier to innovation and growth of the industry, but that the decision to use metallic nanoparticles can be approached with caution.

8.3 Conclusion

This thesis has clearly demonstrated that AgNP exposure can induce an innate immune response in both a monocyte cell line and primary human monocytes by up-regulation of the pro-inflammatory cytokines IL-1, IL-6 and TNF- α . In addition this response was found to involve inflammasome activation. This report has also highlighted the induced toxicity of AgNP upon oral exposure and the vital role of individual biological fluids in modifying toxicity and inflammation. These findings serve to highlight the importance of considering the varied and dynamic environment met by AgNP upon oral exposure and the effects individual components can have on the biological response produced. It also serves to highlight the immunological significance of AgNP following entry to the circulation and the generation of an innate immune response. These findings demonstrate that there is still a long way to go in fully understanding the toxic potential of AgNP following oral exposure and that those responses are varied depending on the biological environment and can potentially contribute to disease development or exacerbate already existing conditions.

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Appendix

UV/VIS absorption spectroscopy was performed to identify the features of cell culture media RPMI and DMEM and of AgNP dispersed in cell culture media and any alterations following the inclusion of the tested biofluid components UDCA, CA, DCA and DPPC. This method was employed to explore potential interaction of AgNP with cell growth medium and its various constituents. The UV/VIS spectrum of cell culture media consists of a number of features at 360, 410 and 560nm. There was also a feature at 270nm not illustrated here. The feature at 410nm was attributed to 10% foetal bovine serum (FBS) added to culture media which also displays another feature at 270nm, the feature at 360nm is riboflavin, a vitamin present in the medium and finally the feature at 560nm can be attributed to the phenol red indicator.

All features can be seen in the absorbance spectra presented (Fig 4.4.1 (a) and 4.4.1 (b)). Because AgNP are dispersed in cell culture media for toxicity testing it is important to establish if their presence leads to interactions with growth factors present in the medium which could play a role in the toxicity of the material. From the data presented the presence of AgNP appears to alter the spectra with a slight increase in absorbance observed upon the dispersion of AgNP in the media and also in the presence of the various biofluid components. Analysis of AgNP alone (dispersed in dH₂O) reveal a feature between the wavelengths of 300 and 400nm which can be attributed to the silver plasmon. There is no evidence of this feature in the preparations of AgNP in cell media and with the addition of biofluid components.

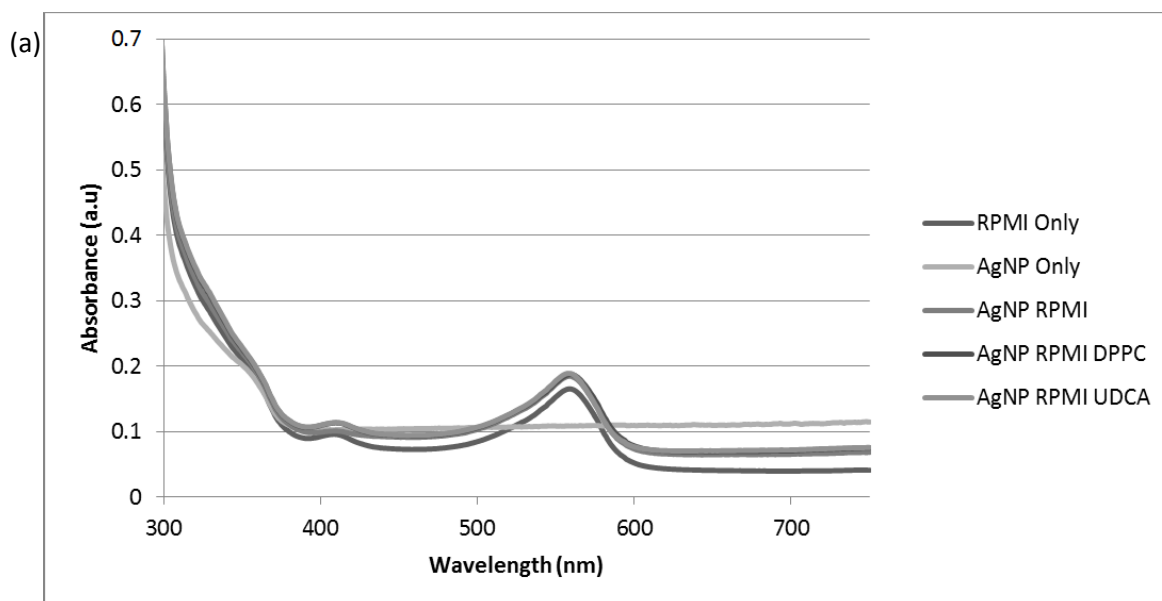


Figure 4.4.1 (a) Absorption spectra of RPMI only, AgNP (15.6 μ g/ml) in dH₂O, dispersed in RPMI, AgNP dispersed in RPMI with UDCA (50 μ M) and RPMI with DPPC (0.25 μ g/ml)

Figure 4.4.1 (a) compares the absorption spectrum of the cell culture medium RPMI, AgNP dispersed in RPMI and AgNP dispersed in RPMI with added DPPC and UDCA. The features of cell culture media as detailed previously at 360, 410 and 560nm are clearly observed from the spectra presented. From the data there was a slight decrease in the peak at 560nm upon the addition of AgNP to RPMI and also with the addition of UDCA and DPPC. There was also an apparent decrease in the peaks and increase in absorbance at 360 and 410nm upon addition of AgNP and biofluid components. The silver plasmon was not apparent upon addition to the different preparations.

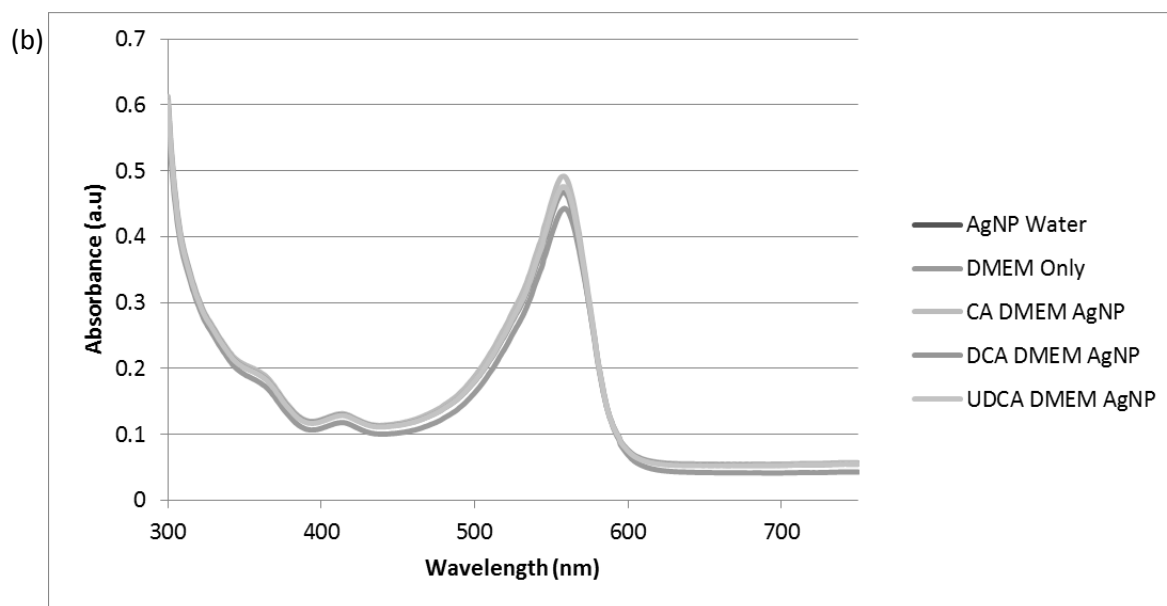


Figure A4.4.1 (b) Absorption spectra of AgNP (15.6µg/ml), DMEM only, and AgNP dispersed in DMEM with CA (1mM), DCA (0.125mM) and UDCA (50µM)

Figure 4.4.1 (b) illustrates a similar spectrum to 4.4.1 (a) but with DMEM cell culture media used as a dispersant. DMEM displays the features at 360, 410 and 560nm, riboflavin, 10% FBS and phenol red respectively. Unlike RPMI these features, particularly the phenol red indicator, are more pronounced in DMEM. As with RPMI a similar alteration to the spectra is observed. A slight reduction in peaks at 360, 410 and 560nm were noted, suggestive of a possible interaction of AgNP with the cell media components. A change in absorbance spectra was also observed in the presence of the biofluid components CA, DCA and UDCA. As before the silver plasmon is not visualised following addition of AgNP to DMEM/ biofluid component preparations.

UV-Vis absorption analysis preparations of AgNP and cell culture media demonstrate slight changes in the absorption spectra. Alterations in spectra can indicate an interaction of AgNP with the molecular components of the culture media. Any interaction could result in a secondary toxicity resulting from depletion of the nutrient and growth factors of the media due to the presence of AgNP. The presence of particles may cause absorption of the media constituents making them unavailable to cells. This has been investigated for other nanomaterials such as carbon nanotubes which resulted in significant depletion of cell culture media constituents possibly contributing to a secondary toxicity by making the various supplements and growth constituents

unavailable to cells. This produced a secondary toxicity as opposed to a primary toxicity (Casey *et al*, 2007). The results demonstrated here show only slight changes in peak width and absorbance values. Although changes in these features can suggest particle-media interaction the changes are so small that any possibility of an interaction is negligible and may not impact on the any overall toxicity in further studies.

Appendix A5.2

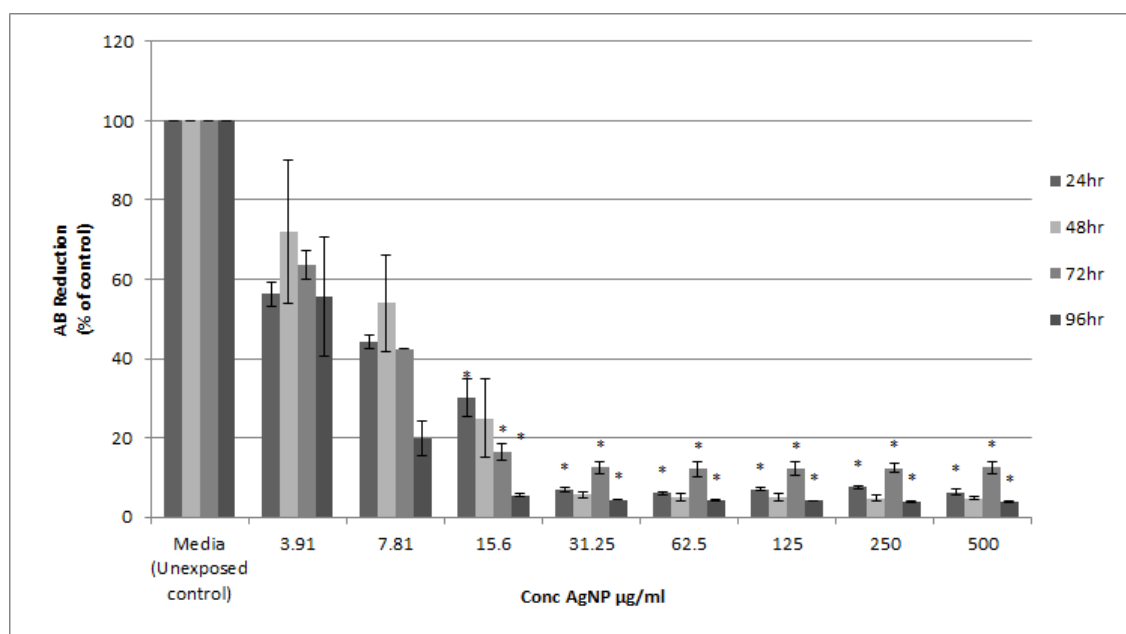


Figure A5.2.1 (a) Cytotoxicity of AgNP in HepG-2 cells after 24, 48, 72 and 96hr exposure as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three individual experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure A5.2.1 represents cytotoxic response of HepG-2 cells to AgNP exposure determined by the AB assay. In the presence of AgNP cell viability decreases in a concentration and time dependant manner. Loss in cell viability is more apparent in the AB assay which assesses the cytosolic integrity of the cell. A reduction in cell viability was observed at the lowest exposure concentrations 3.9 and 7.8 $\mu\text{g/ml}$ after 24 hours. A significant reduction in cell viability compared to the unexposed control was observed at a concentration of 15.6 $\mu\text{g/ml}$ and upwards following 24, 72 and 96 hour exposures

with significant reduction in viability at all exposure time points at concentrations of 31.25µg/ml and above.

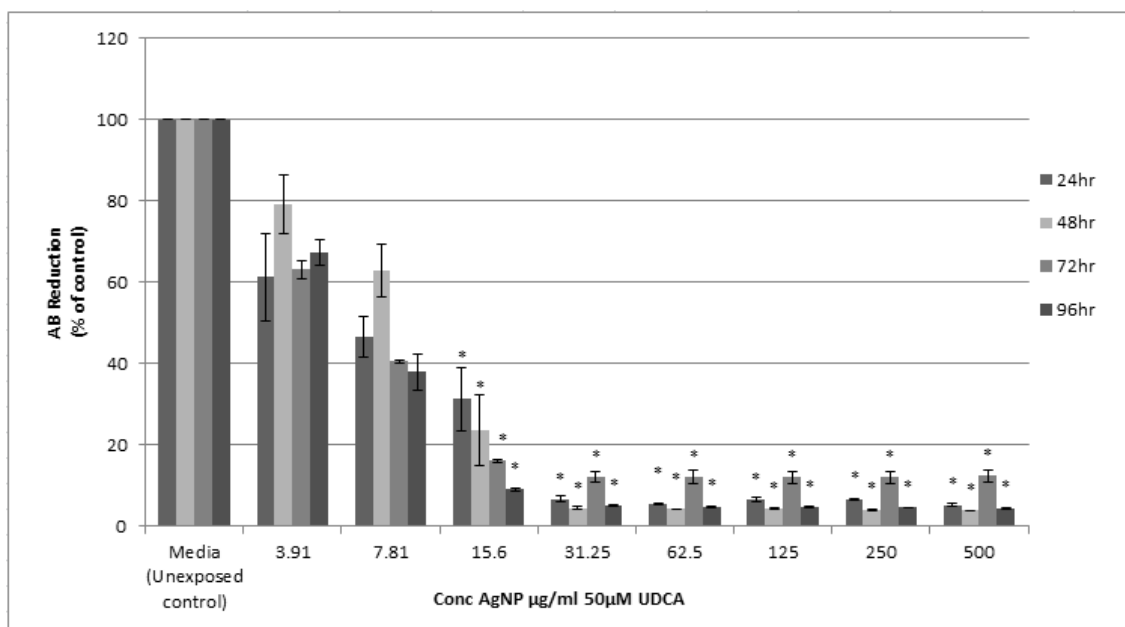


Figure A5.2.1 (b) Cytotoxicity of AgNP in HepG-2 cells with added UDCA after 24, 48, 72 and 96hr exposures as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure A5.2.1 (b) illustrates the toxic response of HepG-2 cells following AgNP exposure in the presence of UDCA determined by the AB assay. A similar concentrationdependent response was noted as seen with the previous assays, following exposure to AgNP alone with the response increasing over time. A decline in cell viability was observed after 24 hours and upwards at concentrations of 3.91 and 7.81µg/ml. A significant decrease in cell viability compared to the unexposed control occurred at all exposure time points at doses of 15.6µg/ml and above. Interestingly the presence of UDCA appeared to have no effect on cellular response to AgNP exposure, with almost identical patterns of toxicity observed with and without the addition of UDCA. This suggests that the presence of UDCA does not modify AgNP toxicity in HepG-2 cells.

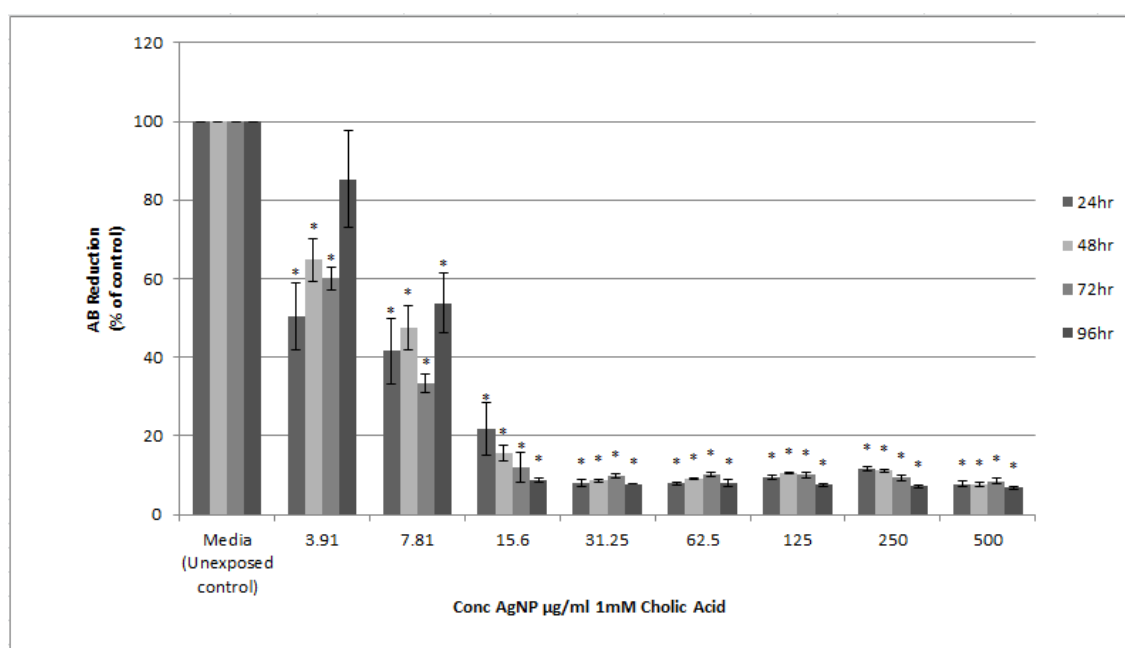


Figure A5.2.1 (c) Cytotoxicity of AgNP in HepG-2 cells with added CA after 24, 48, 72 and 96hr exposures as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure A5.2.1 (c) represents exposure AgNP exposure to HepG-2 cells in the presence of CA. A concentration and time dependant response to AgNP exposure was noted. A significant decrease in cell survival after 24 hours at a concentration of $3.91 \mu\text{g/ml}$ was observed. At this concentration a significant decline in cell survival was detected after 48 and 72 hours. The apparent increase in viability after 96 hour exposure may indicate cell recovery. At this low concentration any surviving cells may begin to replicate as the doubling time for this cell line is 48 hours. A significant decline in cell viability was detected at concentrations of $7.81 \mu\text{g/ml}$ and higher compared to the unexposed control at all exposure time points.

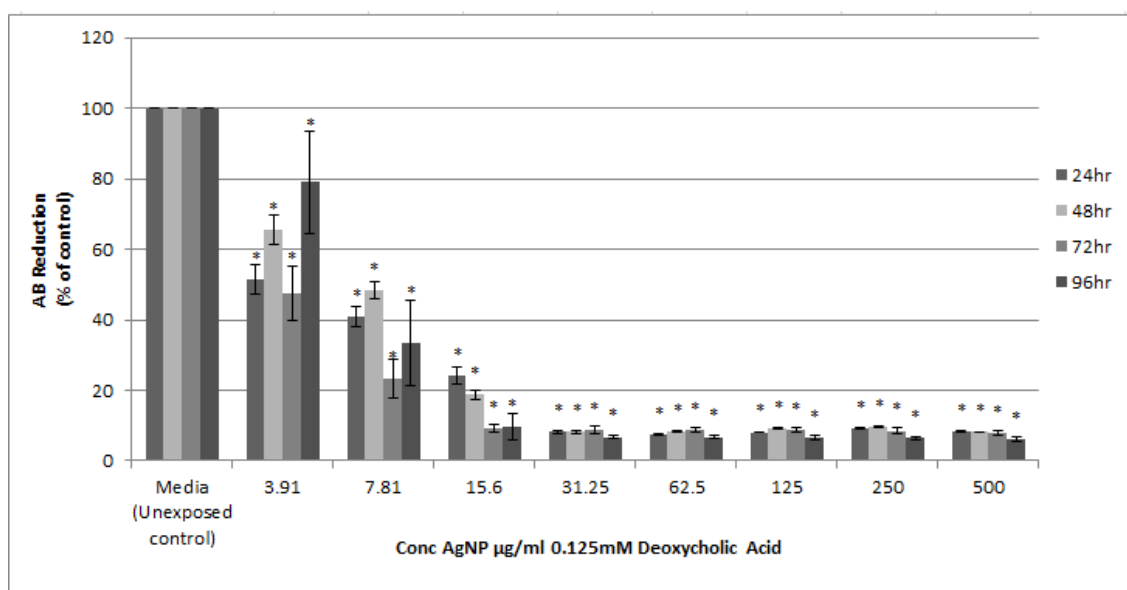


Figure A5.2.1 (d) Cytotoxicity of AgNP in HepG-2 cells with added DCA after 24, 48, 72 and 96hr exposures as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure A5.2.1 (d) illustrates the toxic response of HepG-2 cells to AgNP exposure in the presence of DCA. Exposure resulted in a concentration and time dependent reduction in viability. Significant loss in cell viability compared to the unexposed control was observed at all time points and at all exposure doses.

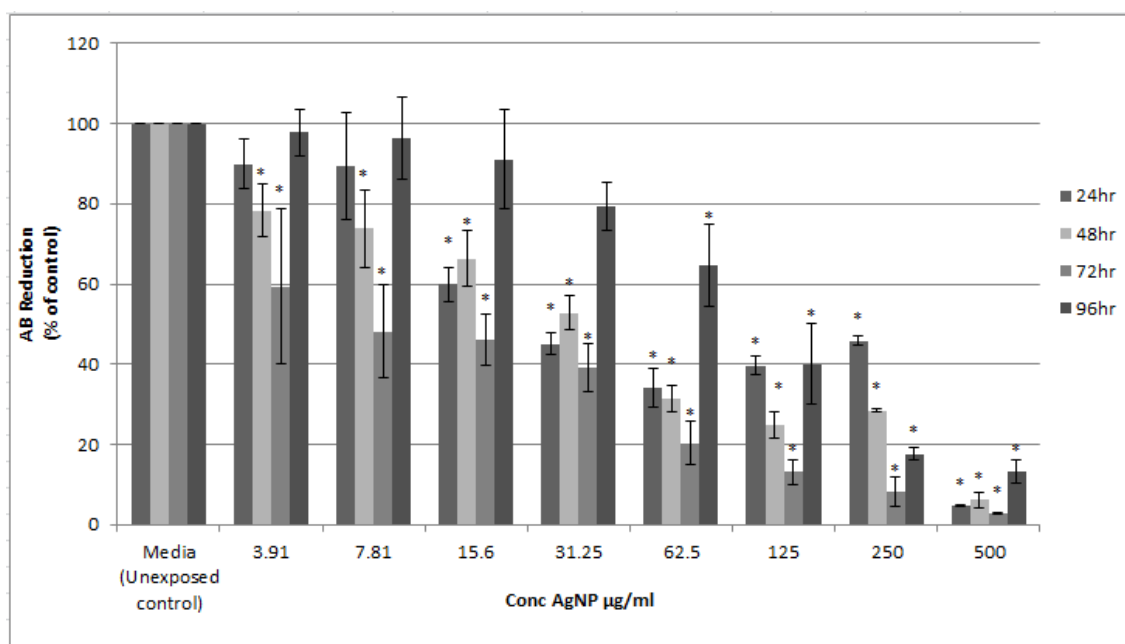


Figure A5.2.2 (a) Cytotoxicity of AgNP to Hep2 cells after 24, 48, 72 and 96hr exposure as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure A5.2.2 (a) displays the cytotoxic response of Hep2 cells to AgNP exposure determined by the AB assay. As with previous cell lines toxicity occurred in a concentration and time dependant pattern. Significant reduction in viability was observed at 48 and 72 hours for all exposure concentrations. A significant reduction in viability compared to the unexposed control after 24 hours was noted at concentrations of 15.6 $\mu\text{g/ml}$ and above with a significant reduction observed for all exposure time points at concentrations of 62.5 $\mu\text{g/ml}$ and upwards. At concentrations of 3.91 to 62.5 $\mu\text{g/ml}$ there appeared to be cell recovery at the 96 hour exposure point. This may be due to remaining particles precipitating out of solution giving any remaining cells an opportunity to begin replicating. With a rapid doubling time of 23 hours cell viability appeared to increase at lower AgNP doses after prolonged exposure.

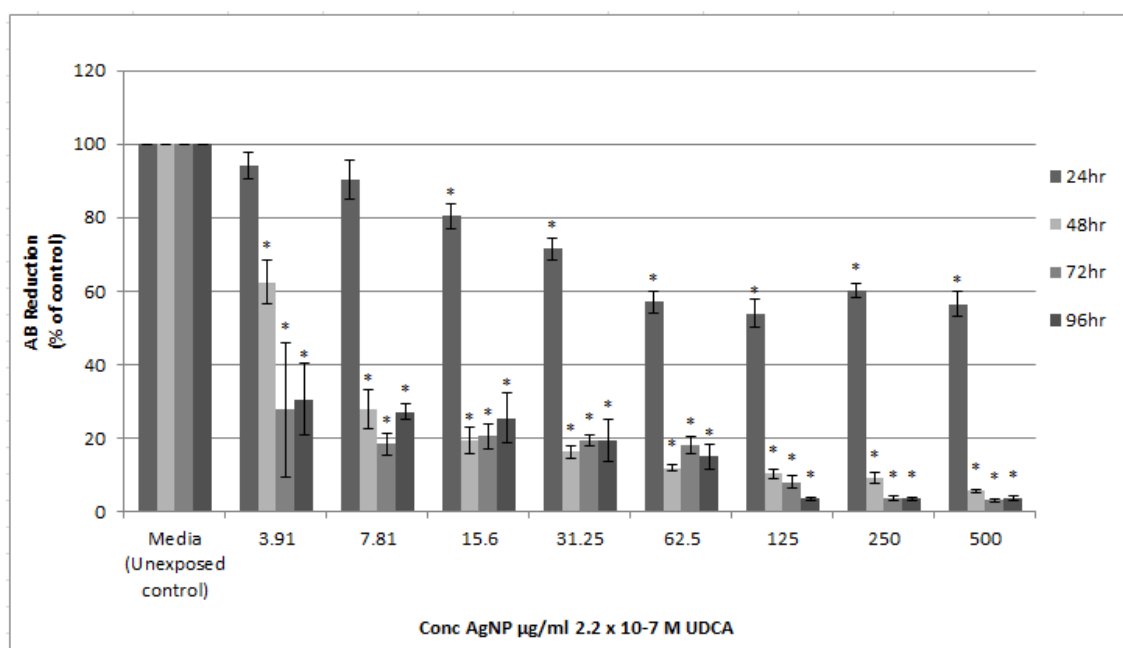


Figure A5.2.2 (b) Cytotoxicity of AgNP to Hep2 cells with added UDCA after 24, 48, 72 and 96hr exposures as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

Figure A5.2.2 (b) illustrates toxic response of Hep2 cells to AgNP in the presence of UDCA. In the presence of UDCA a dramatic reduction in cell viability compared to exposure to AgNP alone was noted. Results demonstrated significant reduction in viability at 48, 72 and 96 hours at all exposure concentrations with a significant reduction at all time points at a concentration of 15.6µg/ml and above. Loss in cell viability was more pronounced following 48, 72 and 96 hour exposures suggesting a possible delay in toxicity. The data presented illustrate that compared to AgNP exposure alone the presence of UDCA does modify toxicity.

Appendix 6.2

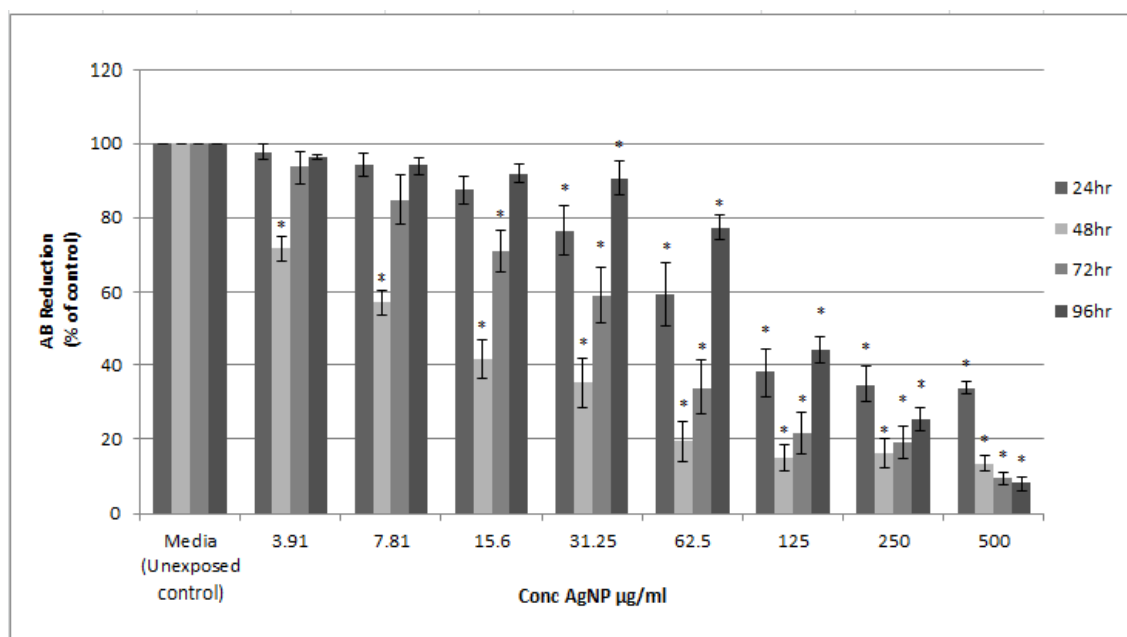


Figure A6.2.1 Cytotoxicity of AgNP to A549 cells after 24, 48, 72 and 96hr exposures as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

The cytotoxic response of A549 cells to AgNP demonstrated by the AB assay is displayed in figure A6.2.1. Decline in cell viability occurred in a concentration and time dependent manner with a significant reduction in cell viability following 48 hour exposures at all AgNP doses. After 72 hour exposure a significant reduction in cell viability was observed at concentrations of 15.6 µg/ml and above with a significant decline at 31.25 µg/ml and upwards at all exposure time points. At 96 hours and 72 hours at lower AgNP doses, cell recovery was noted. It is possible that at this time after exposure and due to the rapid (22 hour) doubling time of A549 cells, the particles have fallen out of solution and with toxic effect having taken place within the first 6-12 hours following exposure. It is possible that any surviving cells begin to replicate at lower exposure doses and at prolonged exposure times. This was observed at concentrations of 3.91-62.5 µg/ml up to 125 µg/ml where a slight recovery was noted. It must also be considered that the sensitivity of viability assays can vary with cell type and while one assay may be suitable for one type a different assay may be more suited to another type.

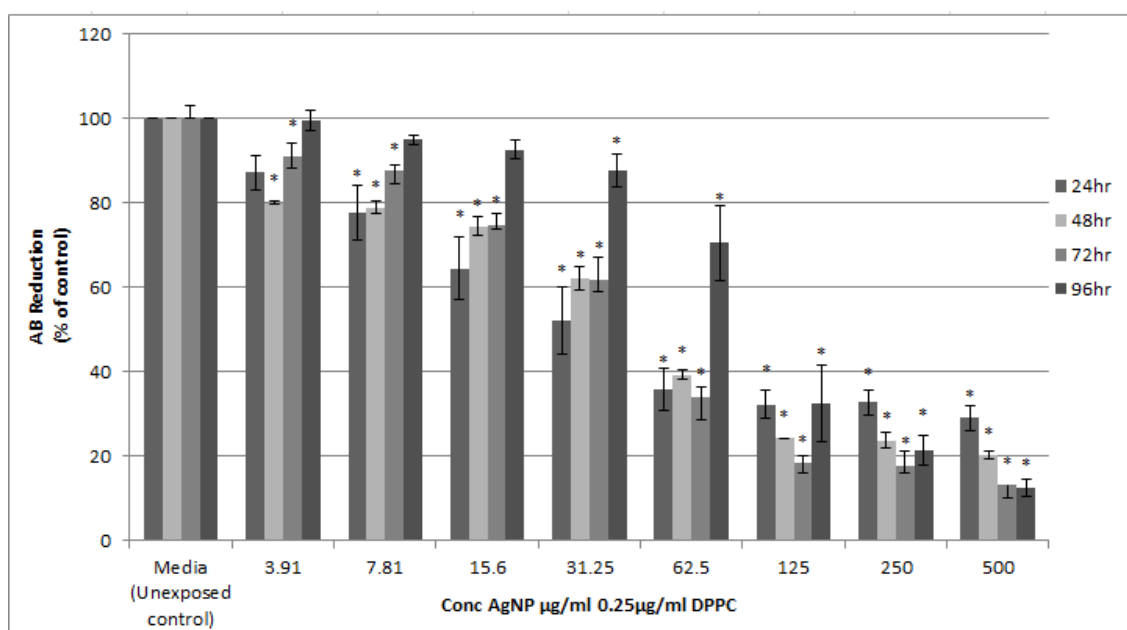


Figure A6.2.2 Cytotoxicity of AgNP to A549 cells with added DPPC after 24, 48, 72 and 96hr exposures as determined by the Alamar Blue assay. Data expressed as percentage of control mean \pm SD of three independent experiments. * denotes a statistically significant ($p < 0.01$) difference from the unexposed control

The response of A549 cells to AgNP exposure in the presence of DPPC demonstrated by the AB assay (figure A6.2.2). A concentration and time dependent reduction in viability was observed. A significant reduction in cell survival compared to the unexposed control was observed at concentrations 7.81 µg/ml and above following 24 hour exposure and at a concentration of 3.9 µg/ml and above after 48 and 72 hours. At 31.25 µg/ml and above a significant reduction in cell survival at all exposure time points was detected. As previously suggested, at lower AgNP doses following 96 hour exposures, the increase in viability may be attributed to cell recovery.

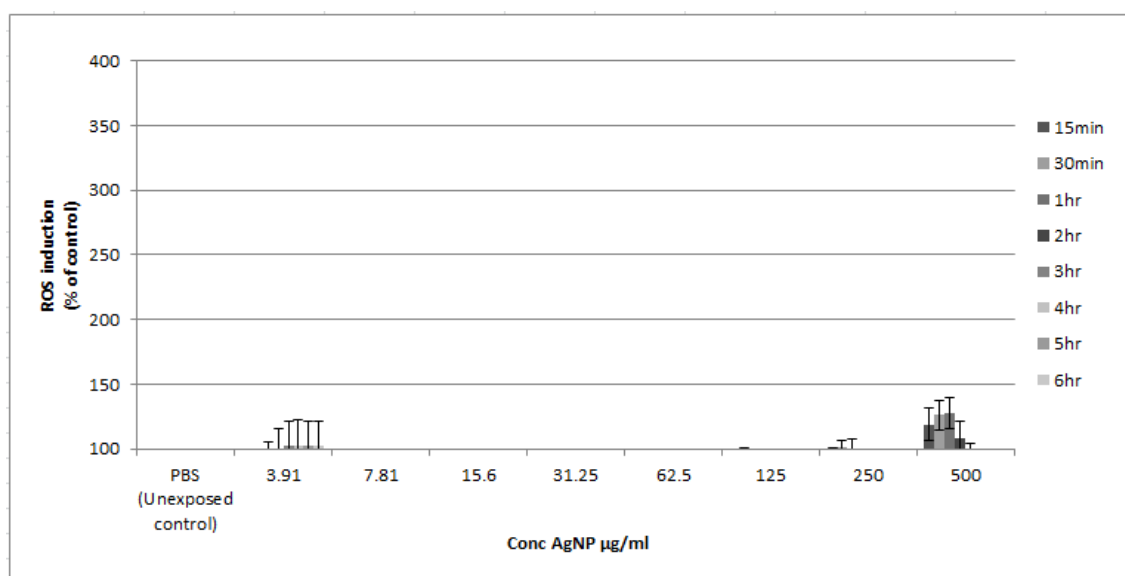


Figure A6.2.3 (a) ROS generation in A549 cells after different time points of AgNP exposure. Data are expressed as percentage of control mean \pm SD of six independent experiments.

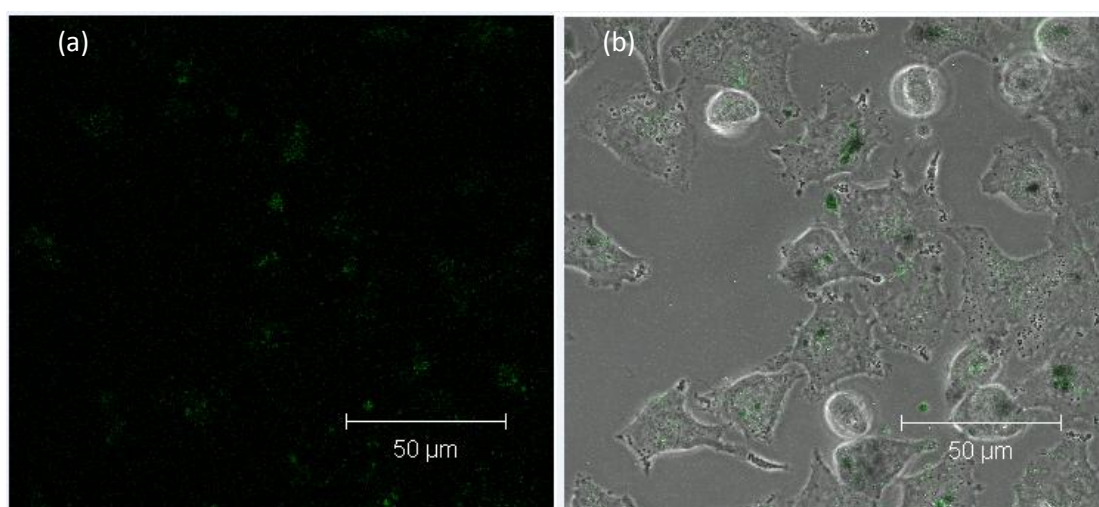


Figure A6.2.3 (b) Confocal image (x40) of A549 cells exposed to 500µg/ml AgNP suspended in cell culture media. Image taken on a Zeiss 510 LSM confocal microscope with an argon ion laser, excitation 488nm using a band pass filter 505-530nm to detect DCF. (a) LSM image demonstrating intracellular ROS, (b) overlay of LSM and bright field image.

The generation of ROS in response to AgNP exposure was determined using 2', 7' Dichlorofluorsecin diacetate which detects intracellular hydroperoxides. Results were expressed as a percentage of the unexposed control. A positive control of 10 μ M H₂O₂ was used in this assay.

Figure A6.2.3 (a) demonstrated little to no intracellular ROS production in response to AgNP exposure. The generation of intracellular hydroperoxides was detected only at the highest concentration, 500 μ g/ml with production increasing in a time dependant manner. ROS production was detected after 15 minutes at the highest AgNP concentration with maximum levels detected after 1 hour. In the hourly readings that followed ROS production rapidly declined. For this particular cell line it was found that AgNP were not potent inducers of ROS. Confirmation of ROS induction by AgNP at a concentration of 500 μ g/ml was verified by confocal microscopy as illustrated in figure A6.2.3 (b).

Appendix 7.2

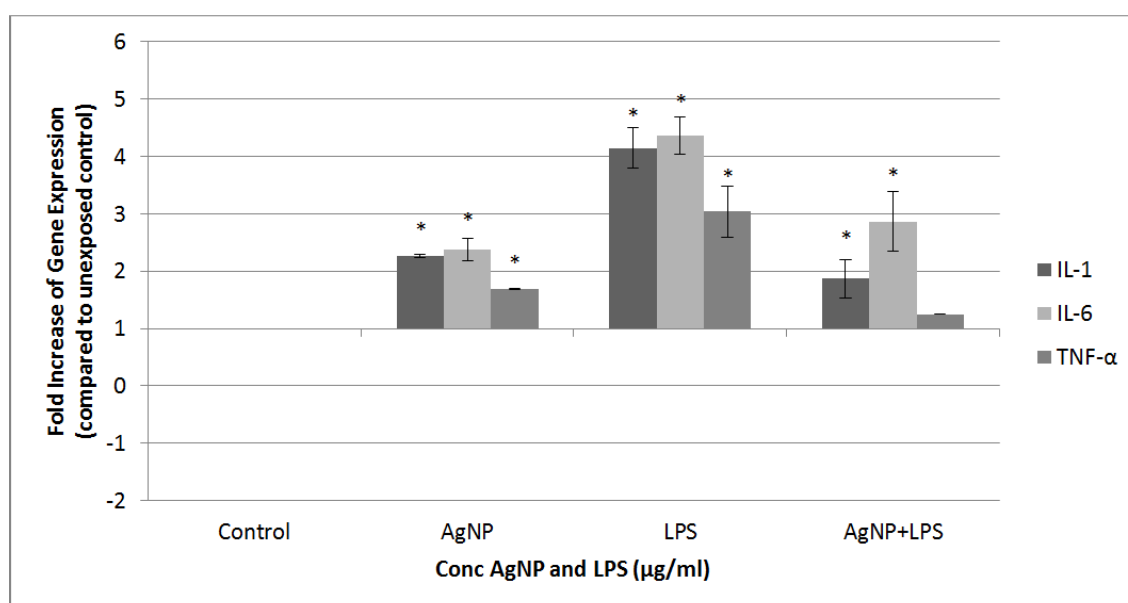


Figure 7.2.1 Fold increase in IL-1, IL-6 and TNF- α gene expression in THP-1 cells following 8 hour exposure to AgNP, LPS and combined AgNP + LPS compared to unexposed control \pm SD of three independent experiments. *denotes a statistically significant difference ($p < 0.05$) from the unexposed control

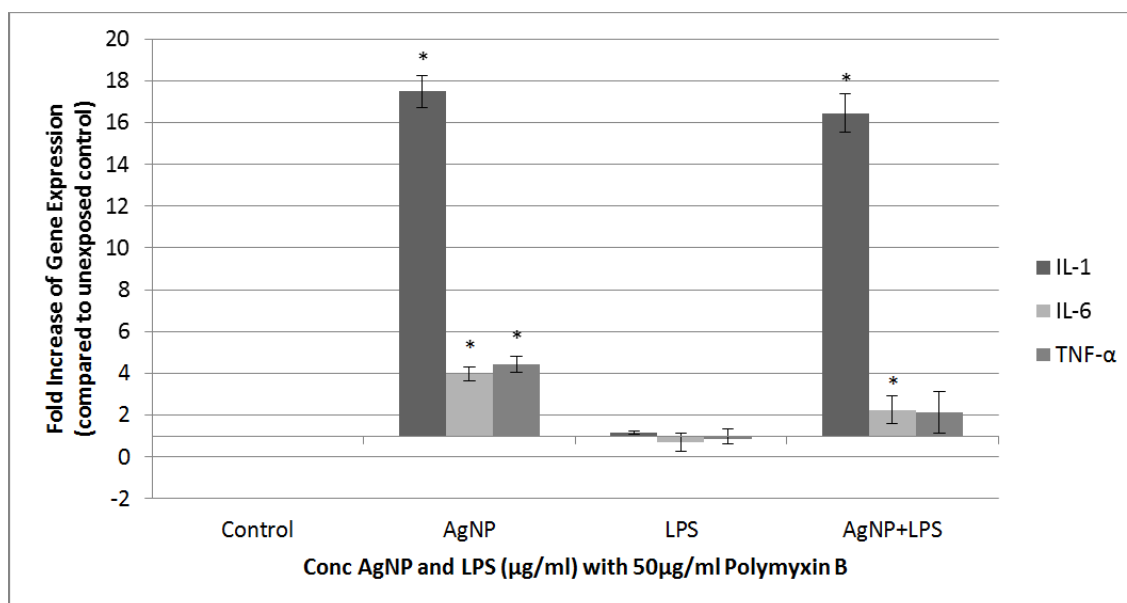


Figure 7.2.2 Fold increase in IL-1, IL-6 and TNF- α gene expression in THP-1 cells following 8 hour exposure to AgNP, LPS and combined AgNP + LPS compared to unexposed control \pm SD of three independent experiments. *denotes a statistically significant difference ($p < 0.05$) from the unexposed control. Polymyxin B (50 μ g/ml) employed as an endotoxin inhibitor was pre-treated 1 hour before exposure.

In order to ensure that gene expression was due to AgNP and LPS exposure alone, polymyxin B an endotoxin inhibitor was pre-treated 1 hour before exposures to ensure no endotoxin contamination in the system. Figure 7.2.1 illustrates pro-inflammatory cytokine gene expression following exposure to 50 μ g/ml AgNP, 1 μ g/ml LPS and a combination of AgNP and LPS. A significant up-regulation in gene expression was observed for all cytokines IL-1, IL-6 and TNF- α following AgNP and LPS exposures. A significant up-regulation in IL-1 and IL-6 was detected following exposure to combined AgNP and LPS. With the addition of polymyxin B (figure 7.2.2) no inhibition of gene expression was observed following exposure to AgNP and combined AgNP and LPS. A significant up-regulation compared to the unexposed control was noted for all cytokine targets following exposure to AgNP. LPS induced gene expression was inhibited by pre-treatment of cells with polymyxin B, however gene expression was still observed following exposure to a combination of AgNP and LPS with significant up-regulation in IL-1 and IL-6 compared to the unexposed control detected. These results demonstrate

no endotoxin contamination in the system and that gene expression is induced by AgNP, LPS and AgNP-LPS exposure alone.

List of Publications

A. Murphy, K. Sheehy, A. Casey, G. Chambers. (2015) Potential of biofluid components to modify silver nanoparticle toxicity. *J Appl Toxicol.* 35 (6), 665-680

A. Murphy, K. Sheehy, A. Casey, G. Chambers. (2015) The surfactant dipalmitoylphosphatidylcholine modifies acute responses in alveolar carcinoma cells in response to low-concentration silver nanoparticle exposure. *J Appl Toxicol.* 35 (10), 1141-1149